# An investigation into differences between indoleacetic acid and fusicoccin in their influence on RNA synthesis, protein synthesis and growth in *Avena* coleoptile tissue.

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(submitted in partial fulfilment of the requirements for the degree of Master of Science)

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St. Catharines, Ontario

January, 1979

To my parents

## Abstract

Growth stimulation of Avena coleoptile tissue by indoleacetic acid (IAA) and fusicoccin (FC) was compared by measuring both their influence on RNA and protein synthesis during IAA or FC stimulated growth. FC stimulated growth more than IAA during the initial four hour exposure, after which the growth rate gradually declined to the control rate. FC, but not IAA, increased the uptake of $^{3}$H-leucine into tissue and the specific radioactivity of extracted protein. Cycloheximide inhibited the incorporation of $^{3}$H-leucine into protein by approximately 60% to 70% in all cases. In the presence of cycloheximide $^{3}$H-radioactivity accumulated in FC-treated tissue, whereas IAA did not seem to influence $^{3}$H-accumulation. These results suggest that FC stimulated leucine uptake into the tissue and that increased specific activity of coleoptile protein is due to increased leucine uptake, not an increased rate of protein synthesis.

There was no measurable influence of IAA and/or FC on RNA and protein synthesis during the initial hours of a growth stimulation. Inhibitors of RNA and protein synthesis, actinomycin D and cycloheximide, respectively, severely inhibited IAA enhanced growth but only partially inhibited FC stimulated growth. The data are consistent with suggestions that a rapidly turning over protein participates in IAA stimulated growth, and that a continual synthesis of RNA and proteins is an absolute requirement for a long term growth response to IAA. On the contrary, FC-stimulated growth exhibited less dependency on the transcription and translation processes. The data are consistent with proposals suggesting different sites of action for FC and IAA stimulated growth.

When compared to $CO_2$-free air, $CO_2$ at 300 ppm had no significant influence on coleoptile growth and protein synthesis in the presence or absence of IAA or FC. Also, 1 mM malate, pH 6.0 did not influence growth of coleoptiles in the presence or absence of IAA. This result was obtained despite reports indicating that 300 ppm $CO_2$ or 1 mM malate stimulates growth and protein synthesis. This lack of difference between $CO_2$-treated and untreated tissue could indicate either that the interstitial space $CO_2$ concentration is not actually different in the two treatments due to significant endogenous respiratory $CO_2$ or else the data would suggest a very loose coupling between dark $CO_2$ fixation and growth.

IAA stimulated the <u>in vivo</u> fixation of $^{14}C$-bicarbonate ($NaH^{14}CO_3$) by about 25% and the addition of cycloheximide caused an inhibition of bicarbonate fixation within 30 min. Cycloheximide has also been reported to inhibit IAA-stimulated $H^+$ excretion. These data are consistent with the acid growth theory and suggest that IAA stimulated growth involves dark $CO_2$ fixation. The roles of dark $CO_2$ fixation in IAA-stimulated growth are discussed.

Acknowledgments

Throughout the course of this research, I have been assisted by a number of persons. I acknowledge the supervision and discussions provided by Dr. A. W. Bown. I deeply appreciate the advice given by Drs. S. Pearce, D. Ursino and P. Nicholls of the department and also thank Professor J. A. Hellebust (Department of Botany, University of Toronto) for discussion of some aspects of my thesis. This work would not have been possible without the generous gift of Fusicoccin from Dr. E. Marré (Centro di Studio del CNR per la Biologia Cellulare e Molecolare delle Piante Instituto di Scienze Botaniche Universita di Milano, Milan, Italy), to whom I express my thanks. I also extend my appreciation to the late J. Findlay (Biology Stores), the technical staff (Glenridge Campus) and my colleagues B. Hill, C. Smith, J. Engemann and T. Koss. I wish to thank J. Hastie for typing this thesis.

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Abbreviations used

| | |
|---|---|
| Act.D | = Actinomycin D |
| CCCP | = carbonylcyanide m-chlorophenyl hydrazone |
| CHI | = cycloheximide |
| $CO_2$ | = carbon dioxide |
| CTAB | = cetyltrimethyl ammonium bromide |
| DCCD | = Dicyclohexylcarbodiimide |
| ER | = endoplasmic reticulum |
| FC | = fusicoccin |
| FCCP | = carbonylcyanide-p-trifluoromethoxyphenyl hydrazone |
| GLP | = growth limiting proteins |
| IAA | = indoleacetic acid |
| KCN | = potassium cyanide |
| OAA | = oxaloacetic acid |
| PEP | = phosphoenolpyruvate |
| PEPC | = phosphoenolpyruvate carboxylase |
| ppm | = parts per million |
| RNA's | = ribonucleic acids |
| SLS | = sodium lauryl sulfate |
| TCA cycle | = tricarboxylic acid cycle |

# INTRODUCTION

The influence of carbon dioxide ($CO_2$) on plant growth and development has been demonstrated for a variety of non-photosynthetic tissues. Three examples may be cited, namely, germination (Negm et al., 1972; Ballard, 1970; Haber and Tolbert, 1959), growth (Splittstoesser, 1966; Bown et al., 1974) and flowering (Langston and Leopold, 1954; Bassi et al., 1975).

In many instances, the influence of $CO_2$ has been related to the dark fixation of $CO_2$ which leads to the formation of 4-carbon acids, e.g., malate and aspartate (Splittstoesser, 1966; Bown and Lampman, 1971, 1972; Dymock, Hill and Bown, 1977; Langston and Leopold, 1954, Ballard, 1970; Yamaki, 1954). Also, speculations have been made for possible roles of dark $CO_2$ fixation in growth of non-photosynthetic tissue such as Avena coleoptiles or tomato roots, etc. (Bown and co-workers, 1974, a, b; 1978; Splittstoesser, 1966; Stout, Johnson and Rayle, 1978; Haschke and Lüttge, 1977b). The present study focuses on the elongation growth of Avena coleoptile tissue, treated with indoleacetic acid (IAA) and fusicoccin (FC) in the presence or absence of 0.03% $CO_2$. The investigation was directed towards a possible relationship between growth and the syntheses of RNA and proteins.

Splittstoesser (1966) reported that the dry weight of tomato roots grown in $CO_2$-free air was forty to fifty percent less than the controls which were grown in air containing 0.03% $CO_2$. The measurements of growth were obtained after five, fifteen and twenty-two-day periods. More recently, the atmospheric level of $CO_2$ (0.03%) or 1 mM malate (pH 7.0) was shown to stimulate the elongation growth rate of Avena coleoptile sections by more than 90% that of the control tissue which was treated with $CO_2$-free air (Bown, Dymock and Aung, 1974; Dymock, Hill and Bown, 1977). In addition, it was

shown that the stimulation of growth by $CO_2$ or malate was observed after a lag of about ten to fifteen minutes and was sensitive to cycloheximide (CHI). In contrast, growth stimulated by a $CO_2$-saturated solution (pH 3.8) exhibited a lag of one min in promoting a 16-fold increase in growth and was insensitive to CHI and other metabolic inhibitors such as KCN, NaF, $HgCl_2$, etc. (Evans et al., 1971). Furthermore, Bown et al. (1974, 1977) reported that in the presence of 20 μm IAA, $CO_2$ or malate exhibited a synergistic effect on growth in Avena coleoptile tissue.

By feeding $^{14}C$-labelled sodium bicarbonate to Avena coleoptile tissue. Bown and Lampman (1971) reported the major fixed products to be malate and aspartate. Similar results, with respect to $CO_2$ fixation, have been obtained with other non-photosynthesic tissue (Splittstoesser, 1966; Poel, 1953; Jacobson, 1955). The presence of 2 μM IAA stimulated the fixation of bicarbonate into malate and aspartate (Bown and Lampman, 1971). Further, either 300 ppm $CO_2$ or 0.5 mM malate was reported to stimulate protein synthesis as measured by the incorporation of $^{14}C$-labelled leucine into protein and also by the levels of soluble protein (Bown and Lampman, 1972; Bown and Aung, 1974). The presence of 20 μM IAA synergistically enhanced $CO_2$-stimulated protein synthesis (Bown and Aung, 1974). Similarly, Splittstoesser (1966) reported a stimulation of protein synthesis by atmospheric $CO_2$ (air) in tomato roots and carrot slices.

Evidence was presented to suggest that the fixation of $CO_2$ may provide an increased supply of 4-carbon acids for the replacement of acids lost to the Krebs cycle in the synthesis of amino acids required for the synthesis of proteins "essential" for growth (Bown and Lampman, 1972, Bown and Aung, 1974, Splittstoesser, 1966). It was also speculated that the fixation of

$CO_2$ and the synthesis of malate may serve to generate hydrogen ions during IAA or FC stimulated $H^+$ excretion and growth (Haschke and Lüttge, 1977b; Hill and Bown, 1978; Stout, Johnson and Rayle, 1978). The accumulated malate may be stored in the vacuole as the divalent cation ($K^+$ or $Na^+$) salt of malate and thus maintain the turgor pressure for the elongating cell (Haschke and Lüttge, 1975, 1977b; Stout, Johnson and Rayle, 1978).

Cycloheximide is widely used to inhibit protein synthesis in eukaryotic organisms. At low concentrations, such as 10 µg/ml, it has been generally accepted to be a specific inhibitor of protein synthesis. It is believed to act on the endoplasmic reticulum (ER) or polyribosomes by interfering with the initiation and transfer reaction in peptide bond formation (Stewart and Letham, 1973; Lüttge et al., 1974; Van Staveninck, 1976; Cocucci and Marré, 1973). On the other hand, the same concentration of CHI has been shown to inhibit IAA-stimulated $H^+$ ion secretion rapidly, followed by the inhibition of growth in Avena coleoptile tissue (Rayle, 1973; Cleland, 1973). Therefore, the inhibition of growth by CHI may be due to the inhibition of $H^+$ ion secretion and/or protein synthesis; also, one might suspect that the blockage of IAA stimulated $H^+$ ion secretion could result in the inhibition of dark $CO_2$ fixation (Stout, Johnson and Rayle, 1978).

In the light of the above information, one may predict that the removal of exogenous $CO_2$ supply may decrease the FC stimulated growth substantially, as it does to IAA stimulated growth (Bown et al., 1974). In addition, FC which stimulates growth and the fixation of bicarbonate into malate, aspartate and glutamate (Johnson and Rayle, 1976) may also stimulate protein synthesis (Bown and Aung, 1974; Splittstoesser, 1966). Therefore, in the present investigation, experiments were designed to perform a comparative

study between auxin (IAA) and the 'superauxin' (FC) under the following headings:

(a) the degree of dependence of IAA and FC stimulated growth on the availability of exogenous $CO_2$ (0.03%),

(b) the effects of IAA, FC and $CO_2$ on RNA and protein syntheses, as measured by the incorporation of the labelled precursors, $^3$H-uridine and $^3$H-leucine, into RNA and proteins, respectively,

(c) the sensitivity of IAA and FC stimulated growth to the inhibitors of RNA and protein syntheses, actinomycin D (ActD) and CHI, respectively,

(d) the effect of CHI on dark $CO_2$ fixation in the presence and absence of IAA.

A Review of the Literature

The pioneering studies on the plant hormone auxin were performed by Charles Darwin and his son Francis about a century ago. In 1881, they published a book entitled "The power of movement in plants" in which they dealt with the phototropic response in grass coleoptiles. It was concluded that an 'influence' produced at the tip was transported to the basal region where it caused the organ to bend (grow) towards light. Nearly fifty years later, in 1928, Went isolated a substance from coleoptiles which possessed similar biological properties to those reported by Darwin. The substance was named auxin. A few years later, in 1934, the chemical structure of the natural auxin was determined as indoleacetic acid (IAA) (Fig. 1). Despite its varied influence on different developmental processes in plants (see Thimann, 1977), auxin's classical effect on elongation growth has been the focus of intense study. These studies have sought to elucidate the mechanism of auxin-stimulated growth.

According to Cherry (1977), the molecular interaction of a hormone with its receptor molecules is referred to as the mechanism of action. The subsequent series of reactions which lead to the observed physiological effect is referred to as the mode of action of the hormone. The present level of understanding of the mechanism and mode of action seems to be fairly well-defined for animal hormones, where a connection has been made between receptor binding and metabolic response (Jensen and Desombre, 1972; Malkinson, 1975). In contrast, hormone action in plants is poorly defined and the literature is riddled with apparent contradictions.

Auxin-induced growth refers to cell elongation resulting from the plastic expansion of the cell wall. It does not involve cell division but

requires the uptake of water to generate turgor pressure, and sugar as a source of energy (Cleland, 1972; Rayle and Cleland, 1977). It also appears to require RNA and protein syntheses (Nooden and Thimann, 1963). At the optimum concentration of 10 to 50 μM IAA (i.e., 2-10 mg/l), and in the presence of 1-2% sucrose, excised auxin-sensitive tissue such as Avena coleoptile grows at a constant stimulated rate for up to 24 h (Thimann, 1977). This may result in an approximate 100% increase in the tissue length while the thickness and density of the cell wall remain fairly constant (Cherry, 1977). Hence, there is little doubt that net cell wall synthesis accompanies the long-term growth response to the hormone. Another striking characteristic of auxin-stimulated growth is the lag period of at least 10 minutes (Evans and Ray, 1969), required before auxin-stimulated elongation is detectable. Evans (1974) suggested that any response to plant hormones detected within 1 h of application be considered as a rapid response. Generally speaking, rapid effects are of interest in considering the mechanism and mode of action of auxin. Several rapid effects involve the cell surface plasmalemma. For example, auxin stimulates the extrusion of hydrogen ions and a transmembranous hyperpolarization, detectable in about 20 min and 7 min, respectively (Rayle and Cleland, 1977; Jacobs and Ray, 1976; Cleland et al., 1977). These processes are closely associated with transport of ions, such as $K^+$, $Cl^-$ etc. (Cleland, 1976b, Marré, 1978, Trewavas, 1976a).

1. The gene activation hypothesis and the effect of auxin on RNA and protein syntheses

The gene activation hypothesis attempts to explain the mechanism of auxin action in terms of altered transcription in the nucleus. The hormone is thought to induce the synthesis of a messenger RNA (m-RNA) which is subsequently translated into an enzyme or a structural protein. This protein is presumed to play an active role in cell wall loosening which makes the wall more extensible. Extensibility is converted into elongation by turgor pressure. The evidence for this hypothesis is based on the following (Ray, 1974):

(a) the effect of inhibitors of RNA and protein synthesis on auxin-induced elongation.

(b) the effect of auxin on RNA and protein synthesis as measured by the incorporation of radioactive labelled precursors (nitrogenous bases or amino acids) into RNA's and proteins, respectively.

(c) auxin's effect on in vitro RNA synthesis as performed by chromatin-directed RNA polymerase systems.

The use of inhibitors of RNA or protein synthesis such as actinomycin D or cycloheximide results in a parallel inhibition of auxin-induced elongation and RNA or protein synthesis (Coartney et al., 1967; Nooden and Thimann, 1966; Penny and Galston, 1966). These data may suggest that specific growth stimulating proteins are synthesized in response to auxin or may simply indicate the requirement for undisturbed RNA and protein syntheses for the hormone response. Therefore, they do not necessarily serve as evidence for the gene activation hypothesis.

Auxin has often, but not always, been shown to stimulate the synthesis of RNA or protein, as measured by the incorporation of radioactive labelled precursors into these polymers. In one isolated case, the stimulatory effect

on RNA synthesis was claimed to have been observed in 10 min after auxin treatment (Masuda and Kamisaka, 1969). Their results have never been reproduced elsewhere. Trewavas (1968) reported that IAA stimulated the incorporation of $^{3}$H-5-orotic acid and U-$^{14}$C-leucine into RNA and protein, respectively, in pea stem sections. More recently, Bown and Aung (1974) had also presented evidence for IAA stimulated protein synthesis in Avena coleoptile sections. In contrast, Venis (1964) had shown that in pea stem sections, IAA reduced the incorporation of $^{14}$C-orotic acid and $^{14}$C-leucine into RNA and protein, respectively. Also, Nooden and Thimann (1963) had presented data that indicated an absence of influence by auxin on protein synthesis in Avena coleoptile tissue.

The major effect of auxin on RNA synthesis seemed to be on the ribosomal RNA (rRNA) fraction (Trewavas, 1968; Ingle and Key, 1965). On the other hand, the synthesis of this particular type of RNA (rRNA) may be completely blocked by the use of 5-fluorouracil without disturbing the auxin-stimulated growth (Key and Ingle, 1964). Therefore, it appears that a major fraction of RNA synthesized in response to auxin is not required for auxin-induced growth. The explanation provided by the authors was that, unlike Actinomycin D which inhibited the DNA-dependent RNA synthesis, 5-fluorouracil could still permit the synthesis of mRNA, which was required for growth. Neumann and Palmer (1971a) have pointed out that a number of reports indicating the stimulation of RNA synthesis by auxin could be due to the metabolism of RNA precursors by contaminating microorganisms on the plant material. They also presented evidence, from experiments with sterile pea stem sections, that the fraction incorporating $^{32}$P-ortho-phosphate into an ethanol precipitate could be successfully separated

from nucleic acids by the combined CM cellulose and sephadex chromatography. In the following report (Neumann, 1971b) the contaminant separated from nucleic acid was identified as a glycerophosphate (MW 5000). It was shown that IAA application stimulated the incorporation of $^{32}$P-orthophosphate into this compound within 5 min, and before the onset of the stimulated growth rate. It was speculated that this compound might serve in a role similar to that of the bacterial cell wall teichoic acids. The teichoic acids are believed to control the ionic concentration of the bacterial cell wall (Heptinstall *et al.*, 1970), and thus regulate the activities of membrane bound enzymes.

O'Brien and associates (1968) reported that the chromatin isolated from auxin-treated soybean hypocotyl tissue, exhibited a large increase in *in vitro* RNA synthesis. The synthesized RNA was fractionated by chromatography on methylated albumin Kieselguhr (MAK) column in an attempt to characterize the tenaciously bound RNA (TB-RNA) fraction, which was thought to be 4s RNA (mRNA). The transcribed RNA's from auxin-treated tissue were shown to stimulate by 100% the incorporation of labelled amino acid ($^{14}$C-leucine) into protein in a cell-free system. However, confirmation from other laboratories is not yet available and to quote Jacobsen (1977), "... it seems to be the only case in plants of *in vitro* translation of cell free synthesized (nuclear) mRNA."

Matthysse and Phillips (1969) isolated a 'soluble fraction', presumably a protein, from pea seedlings. This 'soluble factor' in combination with the added auxin, stimulated the synthesis of RNA in their *in vitro* RNA polymerase system. In contrast, Venis (1971) isolated a hormone binding protein from the same tissue by affinity chromatography

which also promoted the *in vitro* RNA synthesis. Here, auxin was required only during the isolation of the protein factor, but not in the *in vitro* transcribing system. Hardin *et al.* (1972) suggested that auxin caused a release of a plasma-membrane bound 'factor', an 'auxin receptor protein', that moved into the nucleus to stimulate RNA-polymerase activity. There are a few similar reports (Cherry, 1974; Mondal *et al.*, 1972 a, b) supporting Hardin's suggestion of an auxin-released protein which stimulates *in vitro* RNA synthesis. Other investigators (Tautvydas and Galston, 1972) could not find such effects. In short, the differences in details are so great among these reports that none of these isolated 'factors' or auxin receptor proteins appear to be identical (Cleland, 1974), nor do the data seem to be reproducible. Consequently, more questions are left unanswered than are answered.

The removal of IAA from the growth medium resulted in the discontinuation of the elongation response within ten minutes (Evans and Hokanson, 1969). Such an observation may indicate that auxin does not cause a permanent alteration in the cell. If auxin is thought to induce synthesis of a new enzyme(s) resulting in cell wall loosening, the half life of that enzyme is difficult to reconcile with known rates of protein turnover. On the other hand, a rapid inhibition of protein synthesis (3-5 min) and growth (approximately 25 min) (Evans and Ray, 1969; Rayle, 1973) by CHI may be interpreted to indicate a requirement for undistrubed protein synthesis for continued elongation. The time required for inhibition of growth was regarded by some investigators as indicating the pool size of a protein required for growth. The protein was termed the 'growth limiting protein' (GLP) (Cleland, 1971; Penny, 1971). These investigators

suggested that auxin stimulated the synthesis of the GLP, that was thought to be a structural protein (a cell wall protein), although it (GLP) has never been isolated. On the contrary, Pope and Black (1972) had observed IAA-stimulated growth for 8 h under maximal inhibition (>90%) of protein synthesis by CHI, in wheat coleoptile sections. They concluded that protein synthesis was not involved in IAA-promoted growth. A similar conclusion was reached by Addink and Meijer (1972). Nevertheless, evidence has been presented to indicate that IAA or 0.03% $CO_2$ stimulated the incorporation of $^{14}C$-label from leucine into coleoptile protein, and IAA stimulated the incorporation of labelled bicarbonate into protein (Bown and Aung, 1974). These data are consistent with the GLP concept.

With a few exceptions (e.g., Masuda and Kamisaka, 1969), the enhancement of RNA and protein syntheses by auxin was only detected hours after the hormone treatment. Therefore, these effects do not appear to be involved in the rapid stimulation of the growth rate. The characteristic lag period for auxin stimulation of growth (8-15 min, Venis, 1977) seemed to be too short for such macromolecular syntheses. In addition, a pretreatment with Actinomycin D for 60 min, or with cycloheximide for 20 min, which was shown to inhibit RNA and protein syntheses by more than 90% did not influence the lag period (Evans and Ray, 1969). These observations may be taken as evidence against the gene activation hypothesis.

2. The Proton Pump Hypothesis

'The acid growth effect', originally noted by Bonner in 1934, and recently rediscovered and characterized in more detail (Rayle and Cleland, 1970; Evans et al., 1971), refers to the elongation growth caused by

solutions of low pH (pH 3-4), e.g., acidic buffer solutions and $CO_2$ saturated solutions. This effect resembles auxin-induced growth in that both induce similar growth rates and have similar temperature dependencies. Furthermore, the auxin response is abolished in alkaline media (Hager et al., 1971). However, the acid growth effect may be distinguished from the auxin stimulated growth by its lack of sensitivity to metabolic inhibitors such as cyanide and cycloheximide. Also, the acid growth effect has a short lag (<1 min) and the stimulated growth rate is of short duration (<2 h) (Rayle and Cleland, 1970, 1972; Evans et al., 1971).

In 1971, Hager et al. presented a hypothesis concerning the primary action of auxin, by observing the similarities between the acid growth effect and auxin-enhanced growth. According to this hypothesis, which is more commonly known as the proton pump hypothesis or the acid growth theory, auxin activates an outwardly-driven hydrogen ion pump located on the plasma membrane at the expense of ATP, to raise the proton concentration of the cell wall. This may result in the initiation of elongation growth, i.e., the increase in $H^+$ ion concentration in the cell wall compartment could either break acid labile linkages of the cell wall polymers (Rayle and Cleland, 1972) or activate a hydrolytic enzyme that has a low optimum pH to cause cell wall loosening (Hager et al., 1971; Johnson et al., 1974). It may also cause the "hydrogen bond creep" in the cell wall between cellulose fibres and the xyloglucan layer (Albersheim, 1974).

Since the postulation of the acid growth theory, much supporting evidence has appeared in the literature. Also, the discovery of the growth promoting fungal toxin 'fusicoccin' (see p. 26) has resulted in attempts to use it in understanding growth promotion by IAA (Cleland, 1974; 1976;

Dohrmann et al., 1974; Lado et al., 1976a, b). Nevertheless, for continued growth promotion by auxin, undisturbed transcription and translation processes are definitely required and rapid inhibition of $H^+$ excretion by cycloheximide indicates the necessity of protein synthesis for $H^+$ excretion (Rayle, 1973; Cleland 1973).

The fact that auxin or FC stimulated growth is mediated by $H^+$ ion secretion is fairly well established. Inhibitors such as CCCP, FCCP, DCCD, dinitrophenol, CHI, valinomycin etc. which inhibit the $H^+$ ion secretion response also inhibit auxin or FC induced growth. In addition, excretion of $H^+$ ions is specific only for active auxins and fusicoccin (Rayle and Cleland, 1972, 1977; Marré et al., 1973; 1974 a, d; Cleland, 1973; 1975; 1976; Hager et al., 1971; Jacobs and Ray, 1976; Mentze et al., 1977; Johnson et al., 1974, Katsumi, 1976).

Rayle (1973) had reported that in Avena coleoptiles, in the absence of auxin, the optimum pH of the medium for maximum extension rate was approximately 4.8-5.0. Cell wall loosening, as determined by extension growth, was initiated at about 5.8. Therefore, it was reasoned that the pH of the primary cell wall solution should be 5.8 or less to initiate growth and that a drop of 1 pH unit would be required to attain the maximum elongation rate. Work with maize coloeptiles and pea stem sections used a pH microelectrode and demonstrated that IAA treatment resulted in a decrease in free space pH before growth was stimulated (Jacobs and Ray, 1976). Cleland (1976c) provided similar evidence with oat coleoptile sections by using a flat surface pH electrode that rested directly on the tissue. He reported a decrease in pH of the bathing medium within 1 min of FC treatment. IAA treatment resulted in a pH drop within a 14 min period.

These lag times indicated that $H^+$ excretion occurs before or concurrently with growth stimulation and constitute evidence for the acid growth theory. Most of these experiments were performed using "peeled" sections for efficient and accurate pH measurements ("peeled" sections are those from which the cuticle is physically removed or abraded). Nevertheless, the pH of the cell wall space is the critical pH and as yet this has not been measured.

The enhanced excretion of hydrogen ions by IAA and FC is accompanied by a rapid hyperpolarization of the transmembrane electric potential, detected in about 7 min for IAA and 20 sec for FC treated tissue (Cleland et al., 1977). These two processes are closely associated with transport of ions (e.g., $K^+$, $Na^+$, $Cl^-$ etc.), which is another rapid response to the plant growth regulators (Cleland, 1976b; Cleland and Lomax, 1977; Marré et al., 1974; Lado et al., 1976a, b). During the past few years, an accumulating body of literature suggests the importance of ion transport in a number of physiological and metabolic processes influenced by plant hormones (Marré, 1977a, b; 1978; Van Steveninck, 1976; Sutcliffe, 1976; Trewavas, 1976a).

The validity of the proton pump hypothesis may be questioned on the grounds that, in the long run, the auxin-stimulated $H^+$ ion secretion may result in an "overacidification" of the cell wall or vice versa, an "overalkalination" of the cytoplasm, conditions which might become detrimental to the growing plant cells.

With regards to the danger of the "overacidification" of the cell wall solution, it was thought that (Cleland, 1975; Rayle and Cleland, 1977) auxin maintained the cell wall pH around 5.0 (supposedly the optimum

pH for maximum elongation), through a dynamic equilibrium in which active excretion of $H^+$ was balanced by an increasing passive uptake of $H^+$ as the external $H^+$ concentration increased. The cell wall pH of 5.0 was not considered injurious to the plant cell.

If it is assumed that the secretion of $H^+$ ions, caused by auxin, results in a rise in cytoplasmic pH, then a metabolic "pH-stat" mechanism (Davies, 1973; Raven and Smith, 1974; 1976) may be involved to regulate cytoplasmic pH.

A model (Fig. 9) indicating a possible relationship between IAA stimulated $H^+$ excretion and a metabolic pH stat was recently proposed (Haschke and Lüttge, 1977b). The model suggests that a small rise in cytoplasmic pH resulting from $H^+$ excretion would stimulate dark $CO_2$ fixation catalysed by PEP carboxylase and that the consequent accumulation of malic acid would release $H^+$ which would resist increases in cytoplasmic pH. This model also suggests that electroneutrality within the cell is maintained by an exchange of $H^+$ for $K^+$ and that potassium malate accumulates in the vacuole. Evidence for this model includes the accumulation of malate and potassium ions in stoichiometric amounts during auxin-induced growth (Haschke and Lüttge, 1975; 1977a). Such a stimulation of malate synthesis and accumulation and the enhanced uptake of $K^+$ ions (or Rubidium, $Rb^+$ ions) in response to auxin and also to FC were confirmed from other laboratories (Johnson and Rayle, 1976; Stout, Johnson and Rayle, 1978; Cleland, 1976; Cleland and Lomax, 1977; Marré et al., 1974; Lado et al., 1976a, b). Further evidence includes the stimulation of dark fixation of labelled bicarbonate by IAA or FC in which the major labelled product is malate (Bown and Lampman, 1971; Johnson and Rayle, 1976). The enzyme PEP

carboxylase which catalyzes dark fixation has been detected in Avena coloeptile tissue (Bown and Lampman, 1971). It has been demonstrated that the activity of this enzyme increases rapidly as pH increases from pH 7.0 to 8.0 (Hill and Bown, 1978), a response which is consistent with its postulated role in the metabolic pH stat. This may indicate that dark fixation is the metabolic source of $H^+$ for excretion. In addition, reports of $CO_2$ stimulated coleoptile growth (Bown et al., 1974) constitute further evidence for the model. Dark fixation is stimulated by IAA with a lag period of approximately 1 h demonstrating that promotion of fixation is secondary to the more rapid promotion of $H^+$ excretion (Haschke and Lüttge, 1977a).

Apart from a role in $H^+$ generation (Hill and Bown, 1978), two other functions of $CO_2$ fixation in the growth process have been proposed. The accumulation of potassium malate in the vacuole may be important in maintaining turgor in cells which are expanding rapidly through turgor driven extension (Haschke and Lüttge, 1975). In addition, dark fixation may be important in supplying organic acids to the Kreb's cycle to replace those which are removed for the synthesis of amino acids which may be utilized in protein synthesis (Bown and Lampman, 1972; Bown and Aung, 1974).

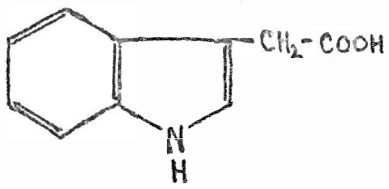
3. Growth stimulation by the 'superauxin' Fusicoccin

Fusicoccin is a fungal toxin isolated from Fusicoccum amygdali, Del., the fungus which causes wilting of leaves in almond trees, Prunus amygdalus St. (Ballio et al., 1964). Thus, it is also known as a wilting toxin. FC has been purified and its diterpene glucoside structure determined (Ballio et al., 1968) (see Fig. 1).

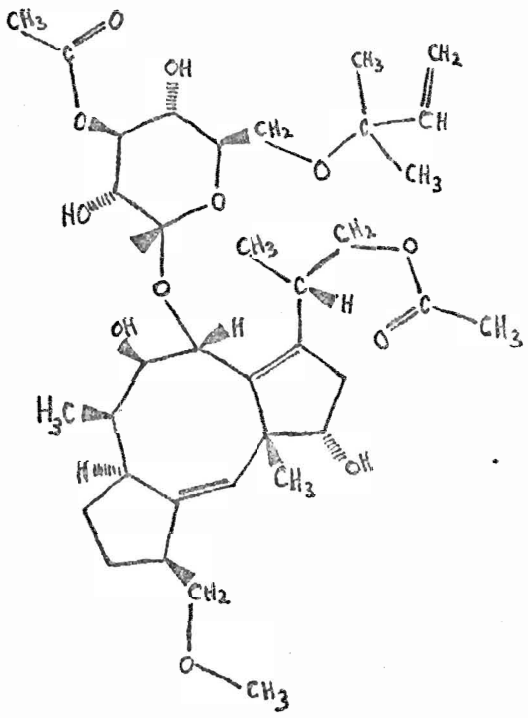
Initially, FC was used to study the mechanism of stomatal opening because of its stimulatory effect on the uptake of $K^+$ ions into guard cells, a process associated with the outward extrusion of hydrogen ions and the opening of stomata (Turner and Graniti, 1969). It was in the early seventies that Marré and his associates declared the discovery of a 'superauxin' by reporting the enhancement of growth and $H^+$ ion excretion by FC in pea stem sections and other plant tissues (Marré et al., 1973 a, b; Lado et al., 1976 a, b and the references cited therein). FC causes greater stimulation of growth, $H^+$ ion secretion, hyperpolarization of the transmembrane electric potential, uptake and fixation of bicarbonate or $CO_2$ and uptake of $K^+$ ions than does IAA treatment. These responses are observed following a shorter lag period than those observed with IAA. Because of these magnified auxin-like characteristics, FC has become a popular tool in studying the mechanism of auxin action (Marré, 1977 a, b; 1978; Cleland, 1974; 1976), although it bears no structural similarity to auxins (Fig. 1).

Figure 1. Molecular structure of IAA and FC

3-Indoleacetic acid




Fusicoccin




(from Ballio et al., 1968)

## Materials and Methods

### 1. Materials

Seeds of Avena sativa, L. var., "Victory" were purchased from Ward's Natural Science Establishment, Rochester, New York, U. S. A. IAA, cycloheximide, actinomycin D, bentonite, CTAB (Cetyltrimethylammonium bromide), tris acetate (trizma acetate) and SLS (sodium lauryl sulfate) were purchased from Sigma Chemical Co., St. Louis, Missouri, U. S. A. Yeast soluble RNA (A-grade) was from Calbiochem, San Diego, California 90054, U. S. A. Glass fibre filters (GF/A, diameter 2.4 cm) were from Whatman Ltd., Maidstone, England. All the radioactive labelled compounds (L-4,5-$^{3}$H-leucine, specific activity 60 Ci/mmol; 6-$^{3}$H-uridine, sp. act. 20.4 Ci/mmol; $^{14}$C-sodium bicarbonate, sp. act. 59.7 mCi/mmol) and the chemicals for the scintillation solutions were from Amersham/Searle Corporation, Oakville, Ontario, Canada. Analyzed gas mixtures were from Linde Specialty Gasses, Oakville, Ontario, Canada. All the other commercial chemicals, which were of analytical grades, were purchased from BDH Chem Co., Toronto, Ontario, Canada; Fisher Scientific Co., Fairlawn, New Jersey, U. S. A. and Canlab, Toronto, Ontario, Canada. Fusicoccin (FC) was a generous gift from Professor E. Marré, Centro di Studio del CNR per la Biologia Cellulare e Molecolare delle Piante, Instituto di Scienze Botaniche Universita di Milano, Milan, Italy.

2. Methods

I. Harvesting and incubation of coeloptile sections and growth measurement

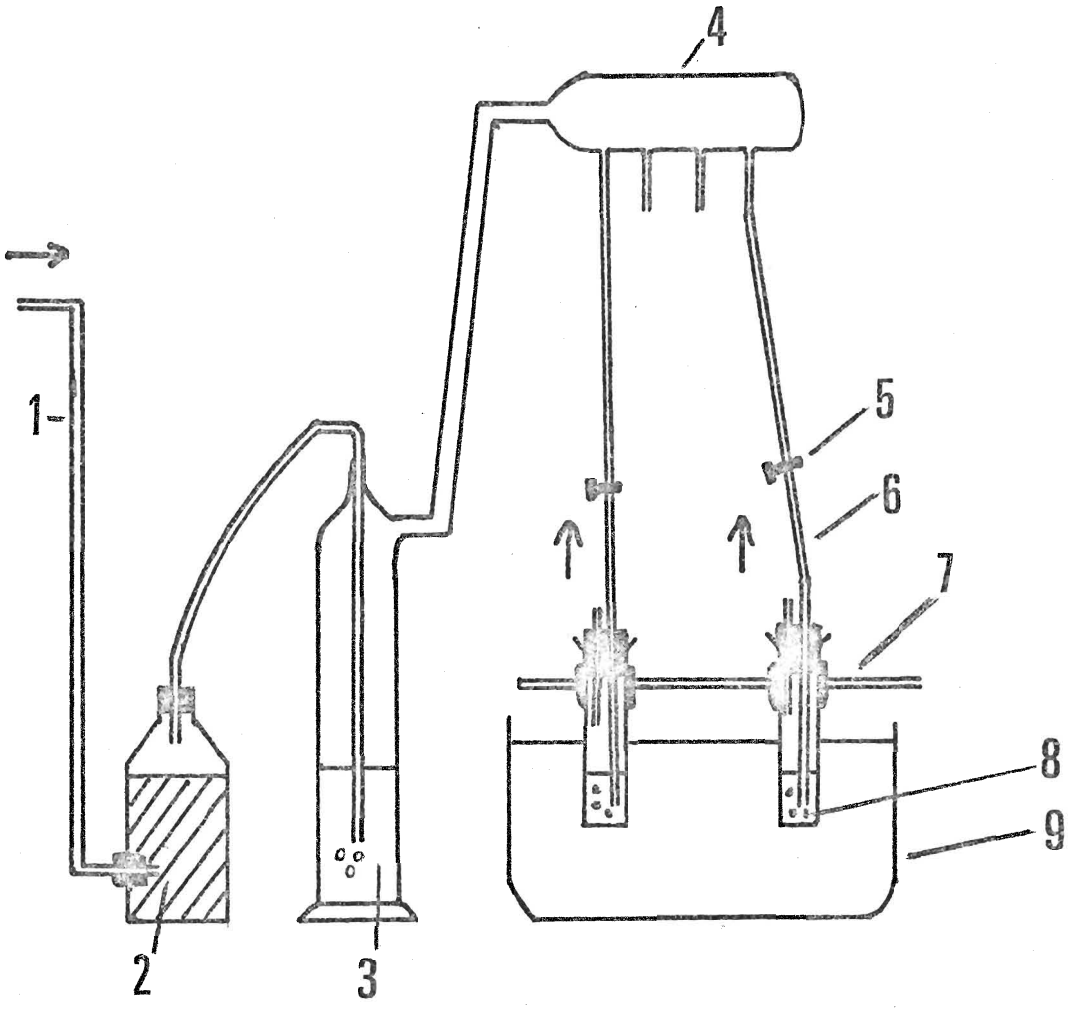
Seeds of Avena sativa, L. variety "Victory" were husked, soaked with stirring in distilled water for about 6 h and then germinated on moist vermiculite in an incubator (Precision Scientific Co. Thelco Model 4) at 25°C under dim red light (a 1 watt neon glow lamp ASA no. JSA, General Electric no. NE30). Three-day old seedlings between 3.2 and 2.5 cm long were used. The 1 cm coleoptile sections (some experiments used 20 mm sections), cut 3 mm below the tip after the removal of the inner leaf, were usually pre-incubated for 45 min in 1 mM pH 6.0 potassium phosphate buffer containing 2% sucrose.

A batch of tissue (consisted of 10 or 20 sections) termed the "zero-hour batch" was photographed for measurement of initial section length, at the end of the pre-incubation period, and was then frozen in liquid nitrogen. At the same time, batches of 10 or 20 sections were placed in test solutions and exposed to analyzed air or $CO_2$-free air for a desired length of time. The test solutions consisted of permutations of 20 µM IAA, 1 mM malate, 1 or 10 µM FC and 10 µg/ml ActD or CHI, dissolved in the potassium phosphate buffer. Radioactive labelled precursors, $^3$H-leucine or $^3$H-uridine, were included in the test solutions. All aerating gasses were passed through a column of distilled water before reaching test solutions. $CO_2$-free compressed air was passed through a bottle filled with $CO_2$-absorber "Indicarb", before passing through water. The tissue was aerated with compressed air nominally 300 ppm $CO_2$ (analyzed grade) unless otherwise noted. A Beckman infrared gas analyzer (Model 215A) was used to check $CO_2$ concentration. The temperature of the incubation tubes was kept constant

Figure 2. Diagram showing tissue incubation apparatus

1. tube from gas cylinder
2. Indicarb
3. water
4. glass chamber
5. clamp for fine adjustment of gas flow
6. aerating tube
7. metal rod from shakers
8. coleoptile sections in test solution
9. water bath (25°C)

Figure 2



at 25°C by use of a water bath, and the tubes were gently agitated by a mechanical shaker (see Fig. 2). Incubation of the tissue was in the dark, except for occasional checks when dim green light was used.

At the end of treatment with test solutions and analyzed gasses, the coleoptile sections were quickly rinsed or washed with distilled water and cold unlabelled solutions (uridine, leucine or sodium bicarbonate) to remove the test solutions and radioactivity. Washing was done in Büchner funnels with the aid of an aspirator. Finally, the sections were photographed, frozen in liquid nitrogen and stored in light-tight vials in the freezer (-20°C). The biochemical data and the growth data were obtained from the same batches of tissue in all the experiments. Growth data was obtained by ptojecting the negative photographic image of the coleoptiles onto a wall using a slide projector and measuring section lengths at approximately 50 fold magnification.

II. Measurement of protein synthesis

Batches of 10 coleoptile sections were incubated in 10 ml test solutions, with aeration, and in the presence of approximately 2.6 μCi of L-4,5-$^{3}$H-leucine (specific activity 60 Ci/mmol). At the end of the incubation period, sections were washed thoroughly with 250 ml of ice-cold unlabelled L-leucine (0.2 mg/ml) and 250 ml of distilled water. All the batches were photographed to measure length and were then frozen in liquid nitrogen and kept in the freezer for protein extraction and assay.

The method employed to determine protein synthesis was that of Dhindsa and Cleland (1975) with a slight modification. The coleoptile sections were boiled for 5 min in 10 ml of 80% ethanol containing 0.2 mg/ml L-leucine.

Soluble radioactivity in the resulting ethanol extract was obtained by counting duplicate 0.1 ml aliquots in 10 ml of ACS scintillation cocktail. The ethanol extract from the "0 h batch", which was not exposed to label, was used to measure background radioactivity. (Almost all the soluble radioactivity was successfully isolated in this first extraction, as the re-extraction with 80% ethanol + 0.2 mg/ml leucine gave a count which was very close to background radioactivity.) After this extraction, the coleoptile sections were treated for 5 min with 10 ml of each of the following: boiling 80% ethanol + 0.2 mg/ml L-leucine; hot 5% trichloroacetic acid; 90% ethanol; absolute ethanol; absolute ethanol + diethyl ether (3:1 v:v) and diethyl ether. After discarding the final solvent diethyl ether, the sections were dried by flushing with a stream of nitrogen gas. The dried sections were then treated for 12 h with 2 ml of 0.2 N NaOH at 37°C in tightly capped vials. The protein content was determined in duplicate using 0.2 ml aliquots, by the procedure of Lowry et al. (1951). Radioactivity in the solubilised protein was determined by use of the liquid scintillation system (Delta 300, Searle Analytic Inc.), counting duplicate 0.1 ml aliquots in 10 ml of ACS scintillation cocktail. Leucine incorporation into protein is expressed as DPM/µg protein. Dhindsa and Cleland (1975) used 1 N NaOH to hydrolyse protein but this concentration was found to be high enough to interfere with the blue colour formation during the determination of protein content.

III. Measurements of RNA synthesis

Batches of 20 coleoptile sections were incubated in 10 ml test solutions, with aeration, and in the presence of approximately 20 µCi

6-$^{3}$H uridine (specific activity 20.4 Ci/mmol). Incubation was terminated by washing with 250 ml of ice cold unlabelled uridine (2 mM) and 250 ml of distilled water. The tissue was photographed to measure growth and then frozen in liquid nitrogen prior to isolation of RNA.

The method of RNA extraction employed was a modification of that used by Verma and Marcus (1973). The frozen coleoptile sections were homogenized using a mortar and a pestle (kept in dry ice), with 2 mg of bentonite. The homogenate powder was transferred to a 30 ml 'corex' centrifuge tube; then 3.5 ml of pH 7.6 TNNE buffer (0.05 M tris acetate, 0.1 M NaCl, 0.1 M sodium acetate, 0.01 M NaEDTA, made 0.5% with sodium lauryl sulfate (SLS)) was added. The suspension was vigorously shaken at room temperature for 1 min, using a vari-whirl mixer. Four ml of phenol-chloroform (1:1 v/v) saturated with 0.01 M pH 8.0 tris-(hydroxymethyl)methylamine (Tris) was then added and the mixture was further shaken for 1-2 min. The mixture was then homogenized in the same corex tube for 3 min using a motor-driven Teflon homogenizer. The aqueous phase was spearated from the phenol phase by a 10 min centrifugation (clinical centrifuge, ≃1500 rpm). After removing the aqueous layer, the phenol phase was re-extracted with 1 ml of pH 7.6 TNNE buffer, made 0.5% with SLS, i.e., shaken for 2 min and then centrifuged to separate the aqueous and phenol phases. Both the aqueous samples were combined and re-extracted with an equal volume of 0.01 M, pH 8.0, Tris-saturated phenol-chloroform (1:1 v/v). The RNA was precipitated from the aqueous phase by adding 2.5 volumes of ice-cold absolute ethanol and storing at -20°C overnight. The resulting precipitate was collected by centrifugation at 0°C, 15,000 g for 10 min. This pellet was vacuum dried in a dessicator at room temperature, dissolved in 5 ml of

distilled water and dialysed against two-one litre changes of pH 7.5 T-K-M buffer (0.01 M Tris, 0.01 M KCl and $5 \times 10^{-4}$ M $MgCl_2$) for 24 h (Ingle, Key and Holm, 1965). The absorption spectrum of the RNA solution was determined by scanning between 300 and 220 nm with a Hitachi-Coleman 124 double beam recording spectrophotometer. It was observed consistently that dialysis resulted in the shifting of the maximum absorbance peak by 10 nm from 270 to 260. The amount of RNA was determined by using the absorbancy at 260 and 290 nm in the following formula:

$$\mu g \text{ RNA/ml} = (OD_{260} - OD_{290}) \times 57 \text{ (Cherry, 1973)}$$

Total RNA (µg) = µg RNA/ml x total ml left after dialysis

Total raadioactivity incorporated into the isolated RNA was determined by the CTAB (Cetyltrimethylammonium bromide) precipitation method (Trewavas, 1967; Bellamy and Ralph, 1968). Briefly, 2 ml of the labelled and dialysed Avena RNA was combined with 10 ml of cold carrier RNA (Yeast RNA, 0.1 mg/ml) and then 1 ml of 1% CTAB solution was added. After standing overnight at 4°C, the RNA precipitate was collected by pouring the suspension onto a glass fibre filter (GF/A), mounted in a suction device to remove supernatant fluid. The filter was washed with distilled water, dried at 45°C for 2h, transferred to a scintillation vial and dissolved in 1 ml of methanol by boiling for 30 seconds. After cooling, 15 ml of scintillation solution, (4 g of PPO (i.e., 2,5-diphenyloxazole) and 0.3 g of POPOP (i.e., 1,4-di-2,5-phenyloxazolylbenzene) in 1 litre of scintillation grade toluene) was added, vigorously shaken and the contents of the vial assayed for $^3H$.

The incorporation of $^{3}H$-uridine into RNA was expressed as DPM/µg RNA. The supernatant fluid (from the CTAB precipitation) had no radioactivity, as indicated by counts which were as low as the background counts. Levels of ethanol soluble radioactivity incorporated into the tissue was determined by boiling the coleoptile sections in 80% ethanol containing 2 mM unlabelled uridine.

IV. Measurement of the incorporation of labelled bicarbonate ($NaH^{14}CO_3$).

In order to investigate the relationship between IAA-stimulated growth and dark fixation of labelled bicarbonate, coleoptile sections were harvested and aerated with air containing 300 ppm $CO_2$ for 45 min at 25°C in 1 mM pH 6.5 potassium phosphate buffer containing 2% sucrose, 1 mM $K_2SO_4$ and 1 mM $CaSO_4$. This solution was reported to result in optimal rates of $H^+$ excretion in coleoptile tissue incubated with IAA (Cleland, 1976c). The tissue was then incubated for various periods of time in the same solution containing various test compounds after which batches of 10 coleoptile sections were incubated for 10 min in air tight capped vials in 2 ml of test solution containing 2 µCi of $^{14}C$ labelled sodium bicarbonate.

The uptake of the label was stopped by the addition of 15 ml of ice cold 0.1 M sodium bicarbonate solution to the vial. The tissue was then quickly rinsed with more bicarbonate solution and more ice cold distilled water. After this, the sections were transferred into scintillation vials containing 2 ml of acidified 90% ethanol (0.01 N HCl) and boiled for 30 sec on a hot plate under the fumehood to remove unfixed $^{14}CO_2$. After cooling, ten ml of ACS scintillation cocktail was added directly to the vials and the radioactivity counted.

V. Radioactive Counting

Radioactive samples were counted in a two-channel Delta-300 Liquid Scintillation System (Model 6890), manufactured by Searle Analytic Inc. All radioactive samples were measured until at least 10,000 counts accumulated. Counting efficiency for each sample was obtained using an external standard system which resulted in a calibration graph that plot the external standard ratio against counting efficiency for a series of quenched standards ($^{3}H$ or $^{14}C$). The counting efficiency obtained for $^{3}H$ samples was approximately 45%, and that for $^{14}C$ samples was about 95%.

VI. Statistical analysis of data

The mean, variance and standard deviation for ungrouped data were calculated as follows:

$$\text{Mean} = (\Sigma X_i)/N$$

$$\text{Variance} = (\Sigma X_i^2 - \frac{(\Sigma X_i)^2}{N})/N$$

$$\text{Standard deviation} = \sqrt{\text{variance}}$$

where N = no. of observations

$X_1, X_2, \ldots X_n$ = observed values

A two-tailed t-statistic was calculated to determine whether or not two sets of data have the same means. The test was performed after demonstrating that the standard deviation of the means were approximately equal.

i.e., $H_0$: $\mu_1 = \mu_2$ where $\sigma_1 = \sigma_2$

$\mu$ = population mean

$\sigma$ = standard deviation

Calculations were performed on a Wang 2200 series computer.

Where statistical analysis has been used, the value of N accompanies the table.

## Results

1. The influence of fusicoccin on growth and incorporation of labelled leucine into protein and into tissue.

Treatment with 10 μM FC stimulated the coleoptile growth rate by more than 5 fold for at least 3 h. The growth rate then returned to that of the control tissue (Fig. 3). The initial rate of growth in FC treated tissue was about 7% per hour, gradually diminishing to about 2% per hour at 4 h, and about 1.2% per hour by 8 h. The growth rate in the control tissue remained fairly constant at approximately 1% per hour throughout the 8 h period.

In the presence of 10 μM FC, the incorporation of $^{3}$H-leucine into protein was stimulated approximately 2 fold (Fig. 4a), However, the extent of stimulation varied as observed in other experiments (see Table I). The increase in specific radioactivity of protein was approximately linear for 4 h. After this, the rate of leucine incorporation was reduced. Consequently, similar trends are observed in growth data and the protein specific activity data.

The level of soluble $^{3}$H-leucine in the tissue, as measured by ethanol soluble radioactivity, was also higher in the presence of 10 μM FC and the leucine uptake was linear with time (Fig. 4b). The increased radioactivity incorporated into protein in the presence of FC was observed after 1 h (Fig. 4a) whereas a substantial increase of label in the ethanol soluble fraction ($^{3}$H-leucine pool) was observed after 2 h (Fig. 4b). The FC-stimulated increase in both the specific radioactivity of protein and uptake of $^{3}$H-leucine into the ethanol soluble pool became increasingly

evident at 4 and 8 h (Fig. 4a, b). It is not clear from the data whether the higher specific radioactivity of protein in FC treated tissue was due to increased uptake of labelled precursor by the tissue or due to an increased rate of protein synthesis.

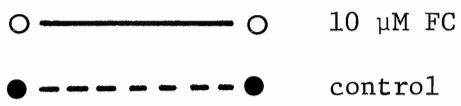
In Table Ia, the stimulation of growth obtained by a 4 h exposure to 1 and 10 μM FC was approximately the same in the absence of CHI. Also, the incorporation of $^{3}H$ leucine into protein in 1 and 10 μM FC-treated tissues was approximately 35% higher than the control tissue (Table Ib). The variability in FC stimulated incorporation of leucine into protein (cf. Fig. 4a and Table Ib) could be due to differences in the amount of $^{3}H$-leucine present in the medium, or to the day-to-day variability of the tissue; for example, see Tables II and III, a and b (Experiments A and B).

CHI completely inhibited the growth of control tissue while it resulted in only partial inhibition of FC-stimulated growth (about 35%) (Table Ia). Furthermore, CHI inhibited the incorporation of $^{3}H$-leucine into protein by about 60-70% in both the control and FC-treated tissues (Table Ib). Both 1 and 10 μM FC stimulated the uptake of $^{3}H$-leucine into the ethanol soluble fraction and CHI appeared to increase this apparent transport of leucine into the tissue (Table Ib). Therefore, it appears probable that the increase in the specific radioactivity of protein in the absence of CHI was due to FC stimulated leucine transport into tissue that resulted in an increase in the specific activity of the leucine precursor pool for protein synthesis. Because 1 and 10 μM FC exhibited effects on growth and $^{3}H$ leucine utilization to an equal extent, all subsequent work was conducted with 1 μM FC.

Figure 3. The effect of treatment with 10 µM FC on coleoptile elongation

Coleoptile sections were pre-incubated in 1 mM, pH 6.0 potassium phosphate buffer (2% sucrose) at 25°C for 45 min. Batches of 10 sections were then incubated in $^{3}H$-leucine with or without 10 µM FC for specified times.

Each data point represents the mean value from four experiments.




Vertical bars represent standard deviation of the mean.

N = 40

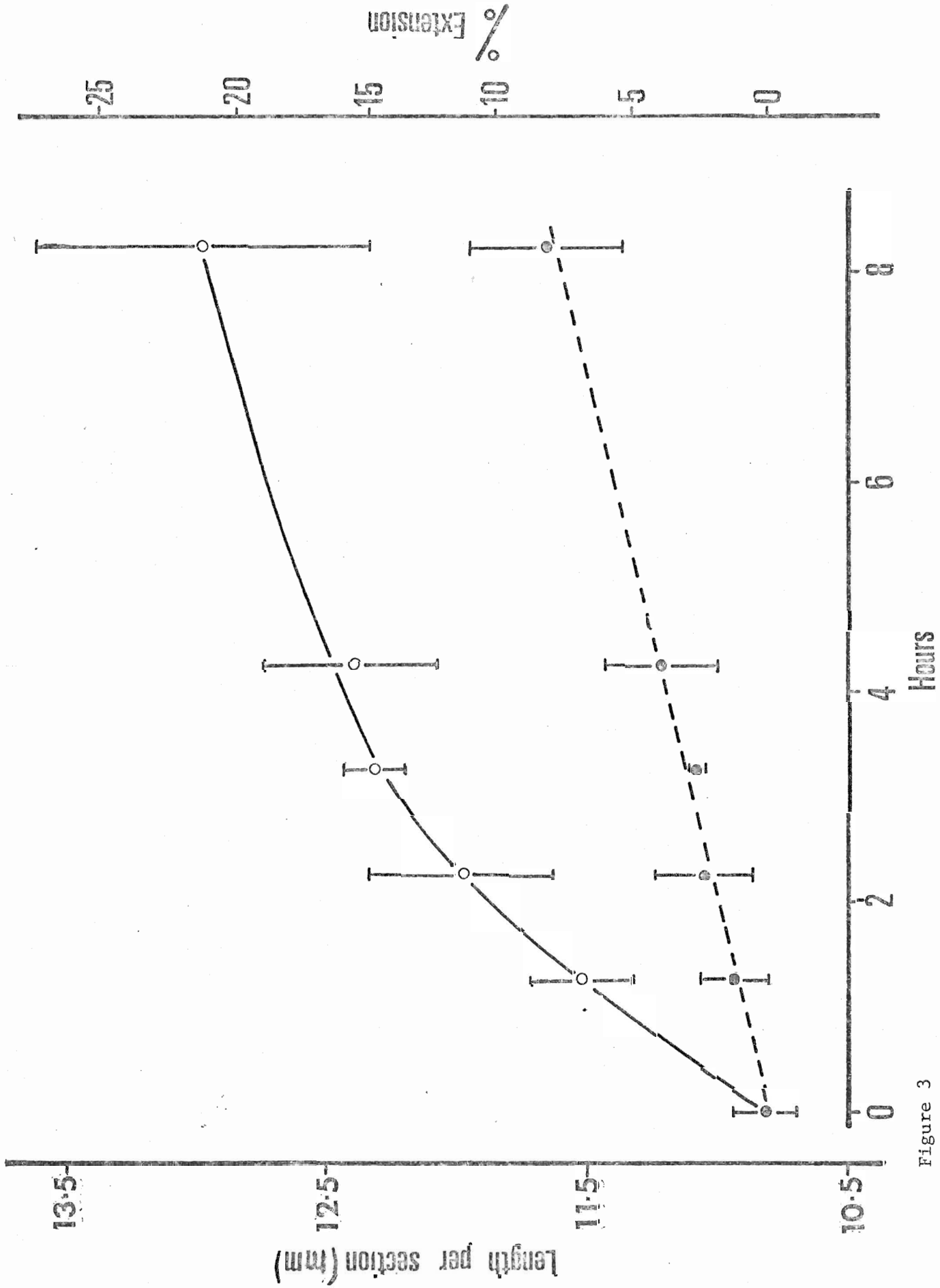

% Extension
25
20
15
10
5
0
Length per section (mm)
13·5
12·5
11·5
10·5
0
2
4
6
8
Hours

Figure 3

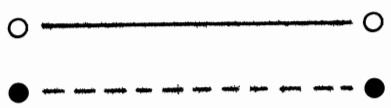
Figures 4(a,b) <u>The effect of 10 µM FC on the incorporation of $^{3}$H-leucine into proteins and on uptake into an ethanol soluble pool</u>.

Coleoptile sections were pre-incubated in 1 mM, pH 6.0 potassium phosphate buffer (2% sucrose) at 25°C for 45 min. Batches of 10 coleoptile sections were then incubated in $^{3}$H-leucine (4.1 µCi) with or without 10 µM FC for specified times. Growth, specific radioactivity of protein and levels of ethanol soluble radioactivity in the tissue were determined.

Each data point represents the mean from 2 experiments. N = 2.

Figure 4(a) shows the effect of 10 µM FC on the specific activity of protein;

Figure 4(b) shows the effect of 10 µM FC on the ethanol soluble radioactivity.



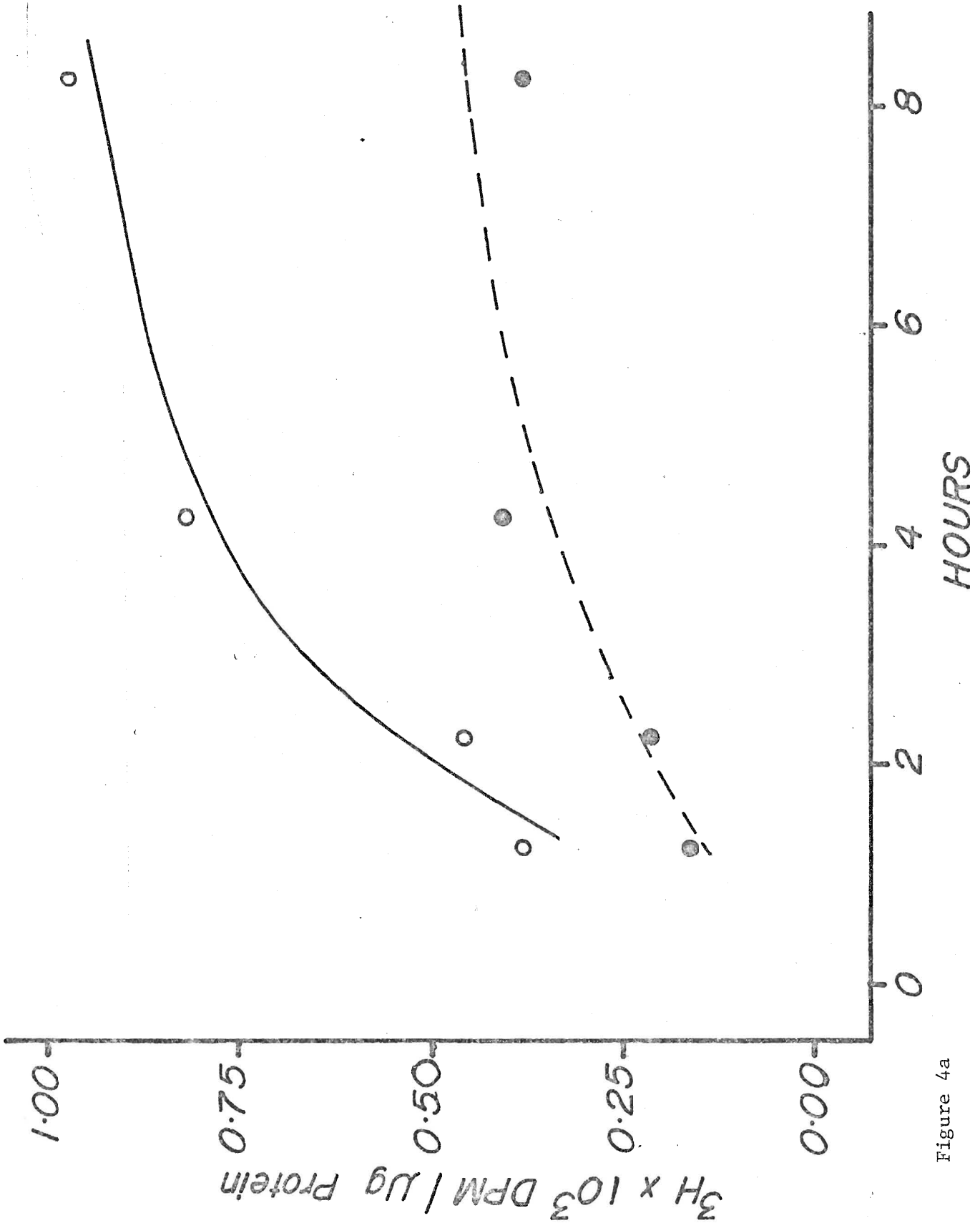



Figure 4a

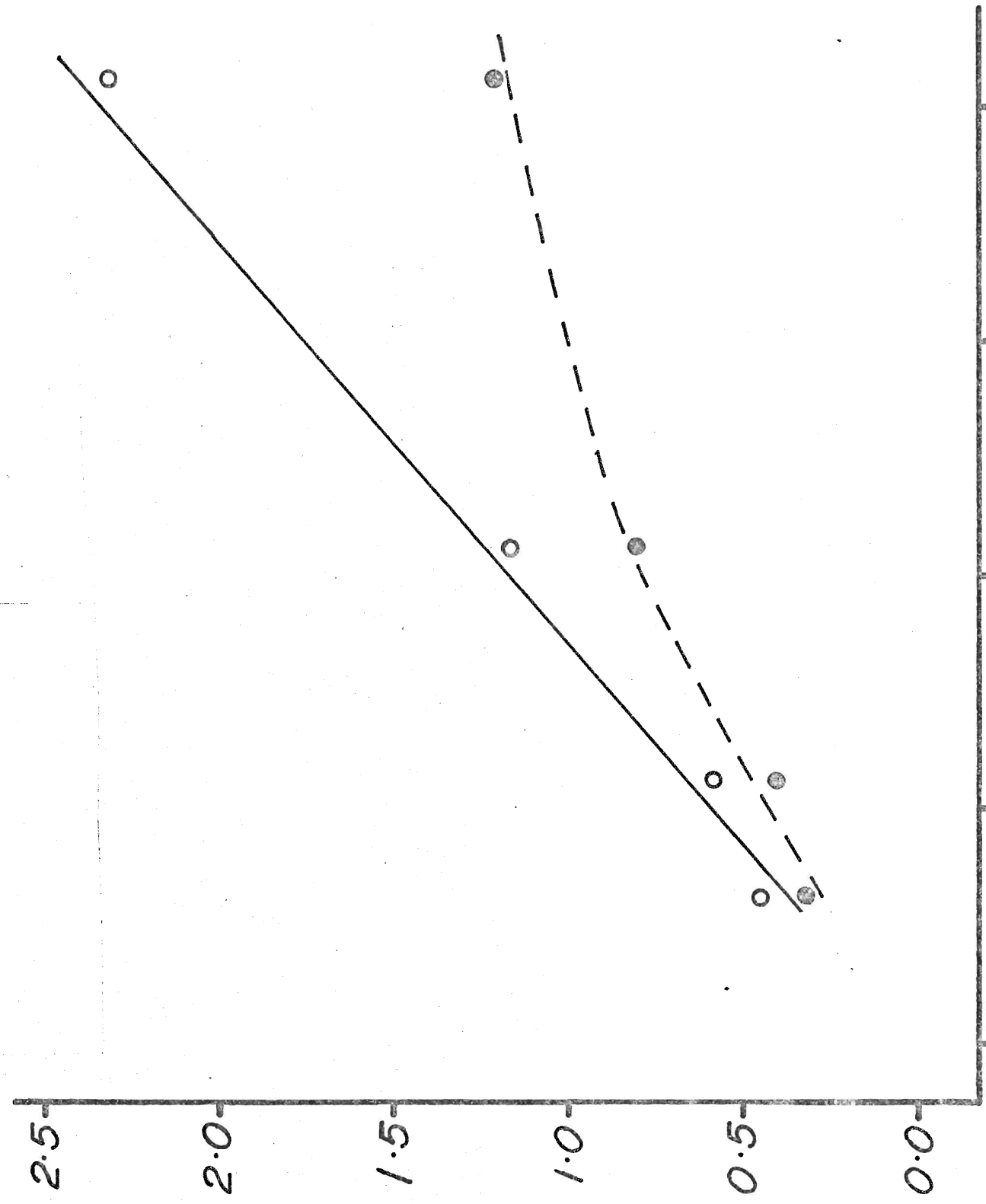

Level of $^3H$-leucine (/10 sections)
$^3H \times 10^5$ DPM
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
HOURS

Figure 4b

Table I(a). The influence of CHI on growth in FC-treated coleoptile sections.

| | | -CHI | +CHI | % Inhibition |
|---|---|---|---|---|
| Control | *Length (S.D.)† | 54.3 (±0.4) | 52.7 (±0.7) | |
| | **% Extension | 3.4 | 0.4 | 88 |
| FC 1 µM | *Length (S.D.)† | 59.6 (±0.6) | 56.8 (±1.3) | |
| | **% Extension | 13.5 | 8.2 | 39 |
| FC 10 µM | *Length (S.D.)† | 58.9 (±0.7) | 57 (±0.5) | |
| | **% Extension | 12 | 8.6 | 28 |

*Length = mean length per coleoptile section in cm, magnified 48 times.

**% Extension is % increase in length over the initial (0 h) length; the initial length was 52.5 (±0.5) cm (i.e., 100).

†(S.D.) is standard deviation of mean. N = 4

See Table I(b) for legend.

Table I(b). The influence of CHI on $^3$H-leucine incorporation into protein and on the levels of ethanol soluble radioactivity

| | -CHI | | | +CHI | | |
|---|---|---|---|---|---|---|
| | Radioactivity ($^3$H x $10^5$ DPM) | | Spec.Act. DPM/µg | Radioactivity ($^3$H x $10^5$ DPM) | | Spec. Act. DPM/µg |
| | Protein | Ethanol Soluble | Protein | Protein | Ethanol Soluble | Protein |
| Control | 1.45 *(±0.14) | 0.85 (±0.13) | 226 (±22) | 0.62 (±0.19) | 0.86 (±0.08) | 97 (±7) |
| 1 µM FC | 1.96 (±0.1) | 1.34 (±0.12) | 306 (±15) | 0.71 (±0.08) | 2.21 (±0.47) | 111 (±12) |
| 10 µM FC | 1.93 (±0.15) | 1.18 (±0.13) | 301 (±23) | 0.56 (±0.07) | 1.91 (±0.33) | 87 (±5) |

* ( ) Values in parentheses are standard deviations of mean. N = 4

Legend for Tables I(a) and (b).

Coleoptile sections were pre-incubated in 1 mM pH 6.0 potassium phosphate buffer (2% sucrose) at 25°C for 90 min. Batches of 10 sections were then incubated in $^3$H-leucine (2.76 µCi) with or without FC (1 and 10 µM) and CHI (10 µg/ml) for a further 4 h. Growth, specific activity of protein and levels of ethanol soluble leucine incorporated into the tissue were determined as outlined in "Methods". Data represent an average from 2 experiments with duplicate batches in each experiment.

2. The influence of IAA and FC.

In the absence of CHI, in Table IIa, the stimulation of growth by 20 μM IAA and/or 1 μM FC was clearly observed. During the 4 h incubation, the degree of stimulation appeared to be higher in FC-treated tissue. IAA had no influence on the specific radioactivity of protein (Table IIa); neither had it any consistent influence on the uptake of $^{3}$H-leucine into tissue (i.e., ethanol soluble radioactivity) nor into protein (Table IIb). In contrast, radioactivity in the ethanol soluble fraction and in protein were considerably higher in FC treated tissue (Table IIa, b). Growth, specific radioactivity of protein and uptake into the ethanol soluble fraction appeared to be highest when both IAA and FC were present (Table IIa and b).

In the presence of CHI, growth was severely inhibited in the control and IAA treated tissue. In contrast, only a partial inhibition of growth resulted when FC was present (Table IIa). On the other hand, CHI inhibited the incorporation of $^{3}$H-leucine into protein to the same level irrespective of the presence of IAA and/or FC (Table IIa, b). In contrast to IAA, FC consistently stimulated the uptake of $^{3}$H-leucine into tissue whether or not CHI was present and an increase in the specific radioactivity of protein was observed in tissue treated with FC in the absence of CHI. But, when protein synthesis was inhibited by CHI, the FC-treated tissue accumulated leucine, as indicated by the ethanol soluble radioactivity. Therefore, it may be concluded that neither FC nor IAA appeared to stimulate protein synthesis in Avena coleoptile tissue, and that the stimulation of the specific radioactivity of protein by FC results from increased leucine uptake.

Table II(a). The influence of CHI on growth and specific radioactivity of protein in coleoptile sections treated with IAA and/or FC.

| | Treatment | Growth % Extension | | | Specific Activity DPM/µg Protein | | |
|---|---|---|---|---|---|---|---|
| | | -CHI | +CHI | % Inhibition | -CHI | +CHI | % Inhibition |
| Expt. A | Control | 4.5 | 1.7 | 62 | 159 | 56 | 65 |
| | IAA | 11.2 | 1.9 | 83 | 156 | 21 | 87 |
| | FC | 14.8 | 13.0 | 12 | 226 | 71 | 69 |
| | IAA + FC | 23.9 | 13.7 | 43 | 270 | 60 | 78 |
| Expt. B | Control | 2.0 | -0.3 | 100 | 60 | 28 | 53 |
| | IAA | 14.9 | -0.2 | 100 | 51 | 27 | 47 |
| | FC | 18.1 | 11.9 | 34 | 76 | 33 | 57 |
| | IAA + FC | 19.4 | 13.9 | 28 | 119 | 32 | 73 |

See Table II(b) for legend.

Table II(b) The influence of CHI on $^{3}H$-leucine incorporation into protein and on the levels of ethanol soluble radioactivity in coleoptile sections treated with IAA and/or FC

| Treatment | Radioactivity $^{3}H \times 10^{5}$ DPM | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Experiment A | | | | Experiment B | | | |
| | -CHI | | +CHI | | -CHI | | +CHI | |
| | Protein | Ethanol Soluble | Protein | Ethanol Soluble | Protein | Ethanol Soluble | Protein | Ethanol Soluble |
| Control | 1.03 | 0.58 | 0.4 | 0.51 | 0.46 | 0.26 | 0.22 | 0.22 |
| IAA | 1.00 | 0.93 | 0.12 | 0.7 | 0.4 | 0.2 | 0.21 | 0.24 |
| FC | 1.5 | 0.86 | 0.48 | 1.04 | 0.58 | 0.58 | 0.26 | 0.7 |
| IAA + FC | 1.8 | 1.16 | 0.36 | 1.5 | 0.92 | 0.73 | 0.24 | 0.82 |

Legend for Table II a and b.

Coleoptile sections were pre-incubated in 1 mM pH 6.0 potassium phosphate buffer (2% sucrose) at 25°C for 45 min. Batches of 10 coleoptile sections were then incubated in permutations of 20 µM IAA, 1 µM FC and 10 µg/ml CHI solutions containing 2.8 µCi $^{3}H$-leucine, for a further 4 h. Growth, specific activity of protein and levels of ethanol soluble radioactivity were determined as described in "Methods".

3. The influence of $CO_2$

The data in Table IIIa and Figure 5 indicate that increasing the exogenous supply of $CO_2$ from zero to 0.03% did not affect growth of IAA and/or FC treated Avena coleoptile tissue, nor did that level of $CO_2$ (0.03%) exert consistent influence on protein synthesis, although results from Experiment B (Table IIIb) seem to indicate a stimulation of protein synthesis by $CO_2$. It may be noted that a marginal increase of radioactivity in protein (Tables IIIa, b) reflected some increase in the ethanol soluble fraction. The data for growth and utilization of $^3$H-leucine in response to IAA and/or FC (in Table IIIb) were consistent with the data in previous experiments (see Tables Ia, b; IIa, b; -CHI columns).

IAA did not seem to stimulate growth in high strength phosphate buffer such as 25 mM, pH 7.5 (Table IV), which was used by Bown et al. (1974) in some of their growth experiments. This is probably due to the preclusion of accumulation of hydrogen ions in the cell wall as explained by Johnson and Rayle (1976) who reported a 90% inhibition of IAA or FC stimulated growth by 10 mM pH 6.2 buffer in "peeled" coleoptile sections. The large standard deviations in means of growth rate (Fig. 5) may be due to the exclusion of sucrose from the medium (Cleland, 1972). Nevertheless, the important point to be made is that 0.03% $CO_2$ failed to stimulate growth in all three buffer solutions used; i.e., 25 mM, pH 7.5 (Table IV); 10 mM, pH 7.0 (Fig. 5); and 1 mM, pH 6.0, 2% sucrose (Table IIIa). A similar absence of influence was also found for malate (Table V).

Figure 5. The influence of treatment with 0.03% $CO_2$ on growth of coleoptile sections in the presence or absence of IAA

Coleoptile sections were pre-incubated in 10 mM, pH 7.0 potassium phosphate buffer at 25°C for 45 min. Batches of 20 coleoptile sections were then incubated in the presence or absence of 0.03% $CO_2$ and/or 20 µM IAA in the same buffer and at the same temperature for specified times.

Each data point represents the mean from 4 experiments.

N = 80

Vertical bars represent standard deviation of the mean.

——————— +IAA

– – – – – – – -IAA

○ $+CO_2$

● $-CO_2$

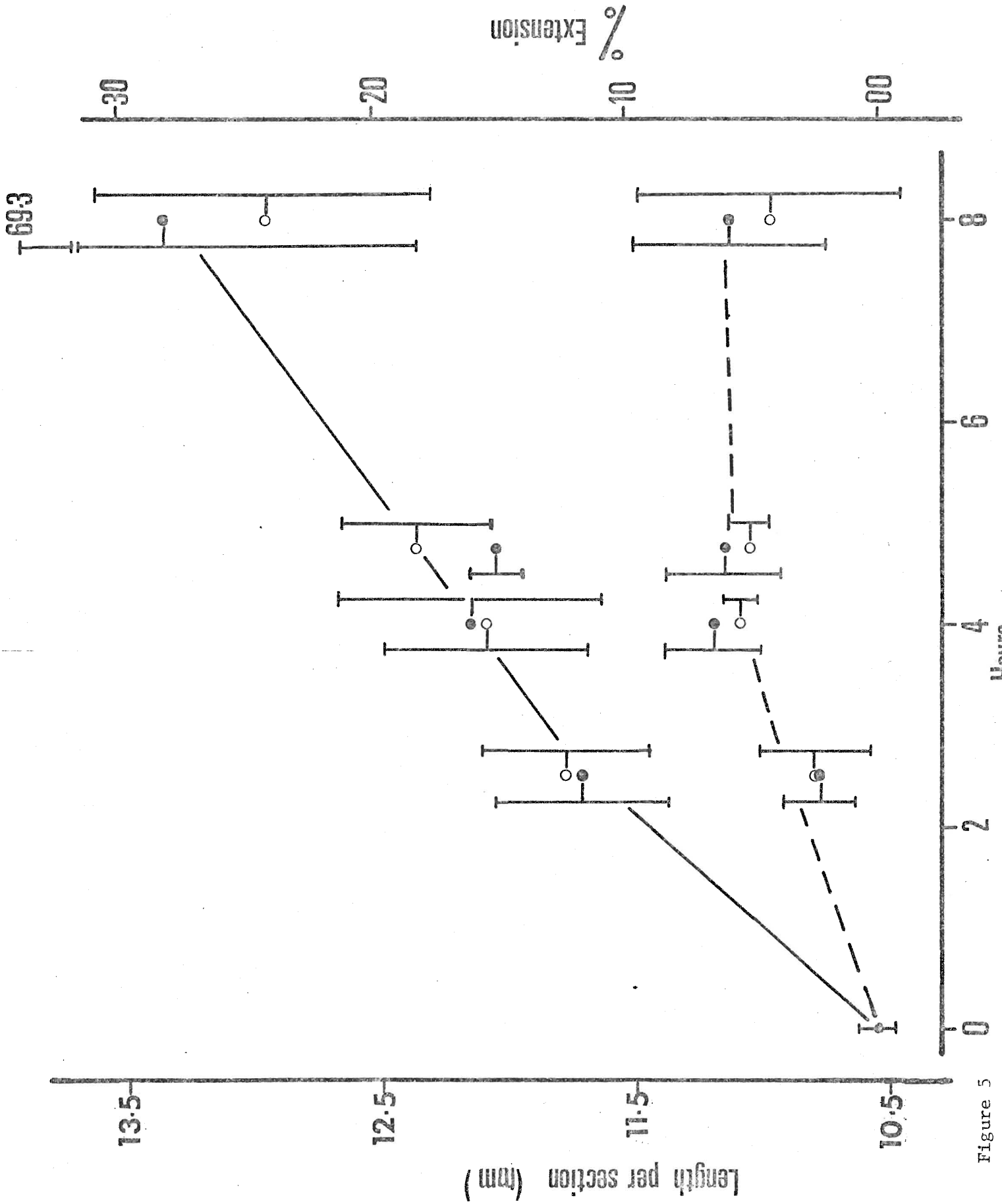

% Extension
30
20
10
00
69·3
Length per section (mm)
13·5
12·5
11·5
10·5
0
2
4
6
8
Hours

Figure 5

Table III(a) The influence of 0.03% $CO_2$ on growth in coleoptile sections treated with IAA and/or FC

| | Treatment | Growth--% extension | | |
|---|---|---|---|---|
| | | $-CO_2$** | $+CO_2$** | Level of significance |
| Experiment A | Control | 4.8 | 2.9 | NS* |
| | IAA | 14.9 | 17.9 | NS |
| | FC | 21.1 | 19.5 | NS |
| | IAA + FC | 22.9 | 23.6 | NS |
| Experiment B | Control | 3.0 | 3.5 | NS |
| | IAA | 16.2 | 15.2 | NS |
| | FC | 22.0 | 22.2 | NS |
| | IAA + FC | 27.3 | 27.1 | NS |

* NS means not significant at P = 0.05, as established by the two-tailed test.

** N = 10

See Table III(b) for legend.

Table III(b) The influence of 0.03% $CO_2$ on the incorporation of $^3H$-leucine into protein and on the levels of ethanol soluble radioactivity in coleoptile sections treated with IAA and/or FC

| | | $-CO_2$ | | | $+CO_2$ | | | |
|---|---|---|---|---|---|---|---|---|
| | Treatment | Radioactivity ($^3H$ x $10^5$ DPM) | | Sp. Act. DPM/µg Protein | Radioactivity ($^3H$ x $10^5$ DPM) | | Sp. Act. DPM/µg Protein | % change in Specific Activity |
| | | Protein | Ethanol | | Protein | Ethanol | | |
| Expt. A | Control | 0.34 | 0.17 | 47 | 0.36 | 0.23 | 51 | +9 |
| | IAA | 0.4 | 0.28 | 56 | 0.4 | 0.27 | 56 | 0 |
| | FC | 0.44 | 0.28 | 62 | 0.54 | 0.42 | 75 | +21 |
| | IAA + FC | 0.78 | 0.62 | 110 | 0.76 | 0.68 | 106 | -4 |
| Expt. B | Control | 0.6 | 0.63 | 92 | 0.77 | 0.66 | 118 | +28 |
| | IAA | 0.68 | 0.67 | 104 | 0.71 | 0.68 | 108 | +4 |
| | FC | 0.86 | 1.1 | 131 | 1.04 | 1.24 | 159 | +21 |
| | IAA + FC | 0.97 | 1.18 | 148 | 1.23 | 1.16 | 188 | +27 |

Legend for Table III a and b

Coleoptile sections were pre-incubated as usual in 1 mM pH 6.0 potassium phosphate buffer (2% sucrose), aerated with the atmospheric air (0.03% $CO_2$) at 25°C for 45 min. Batches of 10 coleoptile sections were then incubated in permutations of 20 µM IAA and 1 µM FC containing 2.6 µCi $^3H$-leucine for a further 4 h. During this period, the batches of coleoptile sections, in 10 ml test solutions, were aerated with or without 0.03% $CO_2$ to obtain permutations of IAA, FC and $CO_2$. Growth, specific activity of protein and levels of ethanol soluble radioactivity were determined as described in the "Methods". Two-tail t-test was used for statistical analysis of growth data.

Table IV. The inhibition of IAA-stimulated growth by high concentration of buffer.

| | Growth--% extension | |
|---|---|---|
| Potassium phosphate | 25 mM (pH 7.5) | 10 mM (pH 7.0) |
| -IAA $-CO_2$ | 1.8 | 4.0 |
| -IAA $+CO_2$ | 0.53 | 2.7 |
| +IAA $-CO_2$ | 1.6 | 10.6 |
| +IAA $+CO_2$ | 1.18 | 10.6 |

Incubation in 25 mM, pH 7.5 phosphate buffer was as described by Bown et al. (1974). Briefly, 6 batches of 10 coleoptile sections (20 mm long, 3 mm below the tip) were pre-incubated in 25 mM pH 7.5 phosphate buffer with or without 20 µM IAA for 1 h (i.e., 3 batches in IAA and 3 batches in buffer only). One batch from both groups of 3 was used to determine the zero hour length at the end of the 1 h pre-incubation; the remaining two batches were used to determine the influence of air or $CO_2$-free air on growth during a 2 h incubation. N = 20

10 mm long coleoptile sections, below 3 mm tip, were used to incubate in 10 mM pH 7.0 phosphate buffer (no sucrose). The zero hour length was measured after a 45 min pre-incubation in the buffer. Batches of 10 coleoptile sections were then incubated in the buffer with or without 20 µM IAA and aerated with air or $CO_2$-free air for an additional 2½ hours. N = 10
The temperature of the bathing medium was 25°C; growth was measured by the photographic technique as described in the "Methods".

Table V. The influence of 1 mM malate and 20 µM IAA on coleoptile growth.

| | % Extension | |
|---|---|---|
| | -IAA | +IAA |
| -malate | 4.27 | 32.07 |
| +malate | 4.39 | 30.72 |

Coleoptile sections were pre-incubated in 1 mM pH 6.0 potassium phosphate buffer (2% sucrose) at 25°C for 45 min. Batches of 10 coleoptile sections were then incubated in 1 mM malate and/or 20 µM IAA in the same buffer and pH for an additional 8 h. Growth was measured as outlined in Methods. Data represent an average from 2 experiments. Aeration was with $CO_2$-free air. N = 10.

4. The influence of IAA and FC on growth and incorporation of labelled uridine into RNA and into tissue.

Dialysis resulted in an increase in the specific radioactivity of RNA and the incorporation of $^{3}$H-uridine into RNA was approximately linear as a function of time (Fig. 6). Consequently, the amount of $^{3}$H-uridine incorporated into RNA after a single fixed time period can be used as a relative indication of the rate of RNA synthesis.

In Table VIa, the stimulation of growth by IAA and/or FC was obvious in the absence of actinomycin D. When tissue was incubated in the presence of IAA or FC for 6 h, greater growth was obtained in the IAA-treated tissue (cf. Tables IIa and IIIa). This result was consistent with the characteristic short duration of FC stimulated growth which lasted no more than 4 h (see Fig. 3) as compared to a constant stimulated growth rate exerted by IAA (Fig. 5). These data are in good agreement with results reported by Cleland (1976a). Furthermore, it became clear from the 6 h incubation that in the presence of FC, IAA could not promote any further growth in addition to the FC stimulated growth (cf. Table IIa and IIIa).

A pre-treatment with actinomycin D for 3 h resulted in a severe inhibition of IAA stimulated growth. In contrast, actinomycin D seemed to cause only a partial inhibition of growth when tissue was treated with FC (Table VIa).

Neither the incorporation of label into RNA nor of label into the ethanol soluble fraction was affected by either IAA or FC (Table VIc). Actinomycin D had no consistent influence on the levels of ethanol soluble radioactivity, although it inhibited the incorporation of $^{3}$H-uridine into RNA.

Figure 6. Incorporation of $^{3}$H-uridine into RNA of coleoptile sections

After a 45 min pre-incubation in 10 mM, pH 7.0 potassium phosphate buffer, batches of 20 coleoptile sections (1 cm long) were incubated in 15 ml medium (20 µM IAA in 10 mM, pH 7.0 potassium phosphate buffer including 19 µCi of 5,6$^{3}$H-uridine specific activity 51 Ci/mmol) at 25°C. At 2, 4, 6 and 8 h, RNA was isolated and the specific radioactivity determined before and after dialysis in TKM buffer, pH 7.4 as outlined in "Methods". Closed circles, dotted lines represent the specific radioactivity before dialysis; opencircles, solid lines represent the specific activity after dialysis.

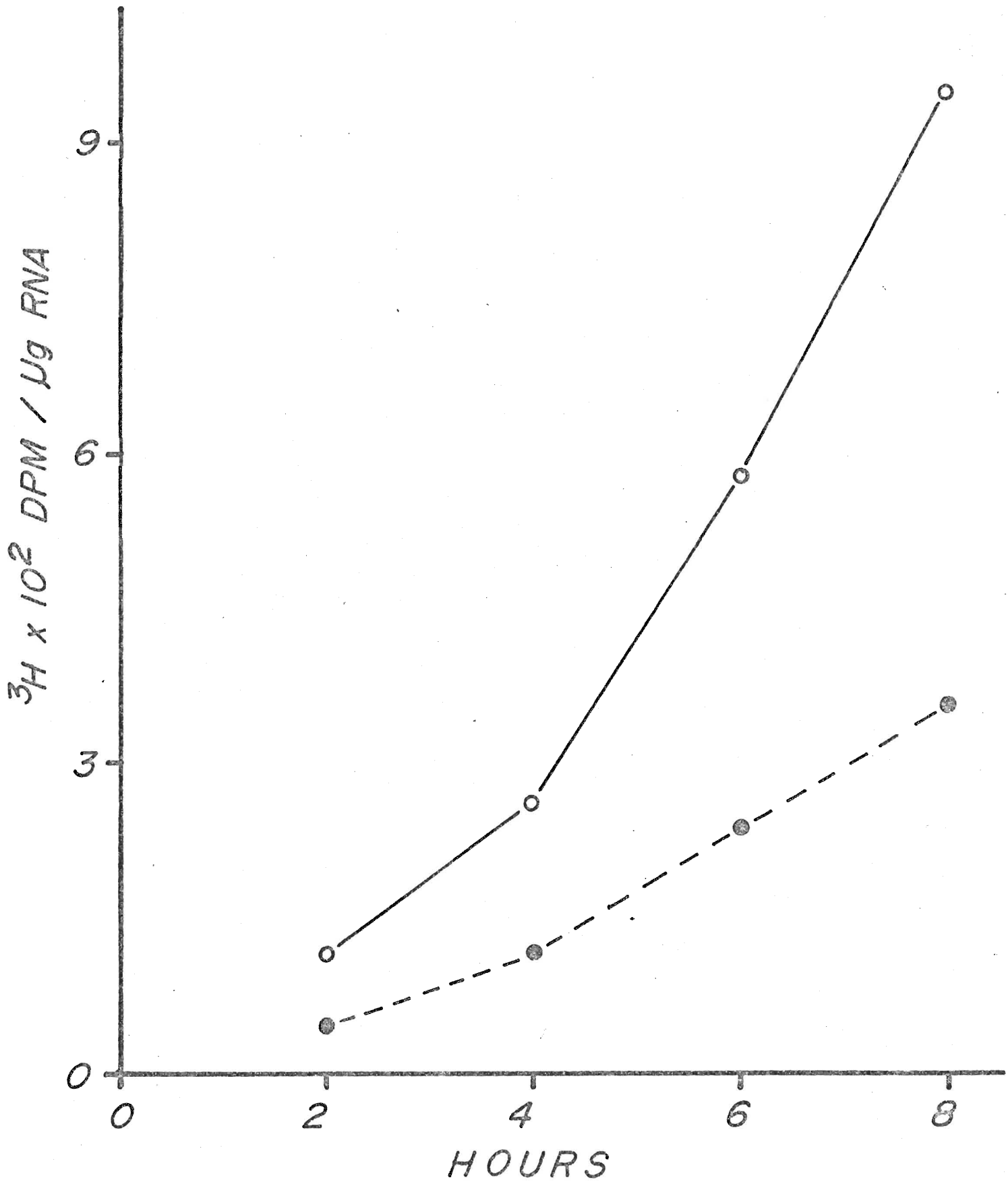



Figure 6

Table VI(a) The effect of Actinomycin D on growth of coleoptile tissue treated with IAA and/or FC.

*Growth--% extension over initial length

| | Experiment I | | | Experiment II | | |
|---|---|---|---|---|---|---|
| | -Act. D | +Act. D | % Inhibition | -Act. D | +Act. D | % Inhibition |
| Control | 12.5 | 7.88 | 37 | 7.17 | 7.34 | 2 |
| IAA | 40.67 | 12.43 | 69 | 31.19 | 12.0 | 62 |
| FC | 36.0 | 20.14 | 44 | 25.07 | 14.0 | 44 |
| IAA + FC | 34.37 | 23.18 | 33 | 22.4 | 19.14 | 15 |

*N = 20

Table VI(b) The effect of Actinomycin D on the specific radioactivity of RNA in tissue treated with IAA and/or FC.

Specific Radioactivity--DPM/μg RNA

| | Experiment I | | | Experiment II | | |
|---|---|---|---|---|---|---|
| | -Act. D | +Act. D | % Inhibition | -Act. D | +Act. D | % Inhibition |
| Control | 6,496 | 1,547 | 76 | 7,993 | 3,307 | 59 |
| IAA | 5,568 | 1,168 | 79 | 7,812 | 4,191 | 46 |
| FC | 6,171 | 1,352 | 78 | 7,031 | 4,593 | 35 |
| IAA + FC | 4,774 | 1,068 | 78 | 5,938 | 2,742 | 54 |

Experimental conditions were as described in the legend under Table VI(c).

Table VI(c) The effect of Actinomycin D on the incorporation of $^{3}$H-uridine into RNA and on the levels of ethanol soluble radioactivity in coleoptile tissue treated with IAA and/or FC.

| | Radioactivity--$^{3}$H x $10^{6}$ DPM | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Experiment I | | | | Experiment II | | | |
| | -Act. D | | +Act. D | | -Act. D | | +Act. D. | |
| | RNA | Ethanol Soluble | RNA | Ethanol Soluble | RNA | Ethanol Soluble | RNA | Ethanol Soluble |
| Control | 0.72 | 2.6 | 0.18 | 1.8 | 0.74 | 1.7 | 0.29 | 1.5 |
| IAA | 0.63 | 2.7 | 0.13 | 2.5 | 0.76 | 1.9 | 0.3 | 1.9 |
| FC | 0.71 | 4.3 | 0.17 | 3.8 | 0.70 | 2.1 | 0.41 | 2.1 |
| IAA + FC | 0.55 | 5.0 | 0.13 | 3.1 | 0.55 | 1.8 | 0.24 | 1.8 |

Coleoptile sections were pre-incubated in 1 mM pH 6.0 potassium phosphate buffer (2% sucrose) for 45 min at 25°C prior to treatment with or without 10 µg/ml Actinomycin D for 3 h. Batches of 27 coleoptile sections were then incubated in permutations of 20 µM IAA, 1 µM FC and 10 µg/ml Actinomycin D solutions containing 20 µCi 6-$^{3}$H-uridine (sp. act. 20.4 Ci/mmol), for a further 6 h. Growth, specific radioactivity of RNA and levels of ethanol soluble radioactivity were determined as described in "Methods".

5. The Influence of IAA or CHI on the rate of fixation of labelled bicarbonate

Coleoptile sections were harvested and aerated with atmospheric air for 45 min at 25°C in 1 mM pH 6.5 potassium phosphate buffer containing 2% sucrose, 1 mM $K_2SO_4$ and 1 mM $CaSO_4$. They were then transferred to the same solution containing 20 µM IAA and incubated for a further 4 h at 25°C. At the end of this incubation, 10 section batches were fed with 0.6 µCi of $^{14}C$-labelled sodium bicarbonate (sp. act. 59.7 mCi/mmol) for 10 sec, 5 min, 10 min, 15 min and 20 min to measure fixation of bicarbonate as outlined in the Methods section. The fixation of bicarbonate increased linearly with time (Fig. 7).

The data in Figure 8 show the influence of CHI on the fixation of bicarbonate. Two batches of coleoptile tissue were incubated in phosphate buffer (1 mM, pH 6.5 potassium phosphate, 2% sucrose, 1 mM $K_2SO_4$ and 1 mM $CaSO_4$) with or without 20 µM IAA for 3 h. The two batches of tissue were divided at 0 h and CHI added to give all 4 permutations of ±IAA and ±CHI. Fixation of sodium bicarbonate was then measured at 0.5 h, 1 h, 2 h and 4 h by removing tissue samples from the 4 batches and pulsing with $NaH^{14}CO_3$ for 10 min (see Methods).

In the absence of CHI, IAA stimulated the rate of $CO_2$ fixation by approximately 25% over the control rate (i.e., -IAA, -CHI). The addition of CHI reduced the rate of dark carbon fixation within 30 min to the same rate as the non-IAA treated tissue. At 4 h, the rates of $CO_2$ fixation of CHI-treated tissue were only 50% that of the control rate.

Figure 7 Kinetics of dark fixation of $NaH^{14}CO_3$ in coleoptile sections

Coleoptile sections were pre-incubated for 45 min at 25°C in 1 mM, pH 6.5 potassium phosphate buffer (including 2% sucrose, 1 mM $K_2SO_4$ and 1 mM $CaSO_4$). The sections were then transferred into a beaker containing 20 µM IAA in the same solution and incubated for a further 4 h. At the end of this incubation, duplicate batches of 10 sections were fed with $NaH^{14}CO_3$ for 10 sec, 5 min, 10 min, 15 min and 20 min. Fixation of $NaH^{14}CO_3$ was determined as outlined in the "Methods".

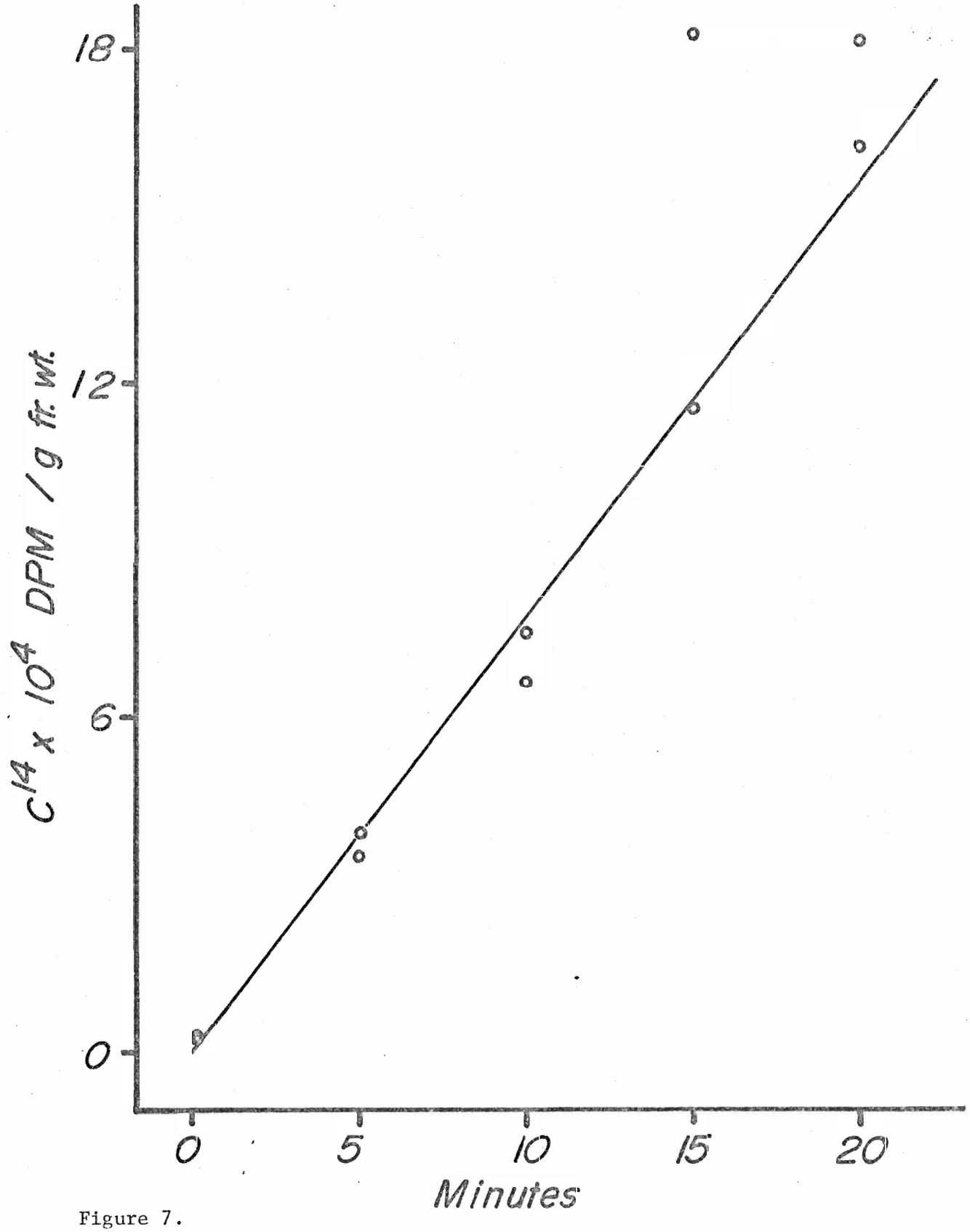
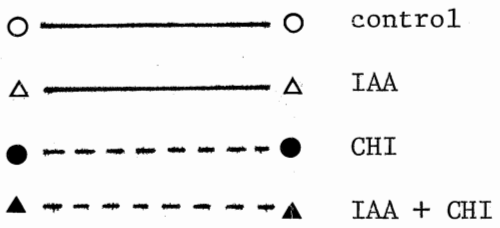



Figure 7.

Figure 8. The effect of CHI on the rate of dark $CO_2$ fixation in coleoptile sections

Coleoptile sections were pre-incubated for 45 min at 25°C in 1 mM, pH 6.5 potassium phosphate buffer (including 2% sucrose, 1 mM $K_2SO_4$ and 1 mM $CaSO_4$). The tissue was then divided into two batches and incubated in the same buffer with or without IAA for 3h. At 0 h, CHI was introduced (final concentration 10 µg/ml) to obtain the four permutations of IAA and CHI. Fixation of $NaH^{14}CO_3$ was determined for each condition at 0.5 h, 1 h, 2 h and 4 h, using a 10 min incubation period. The data represent the mean value from 2 experiments.



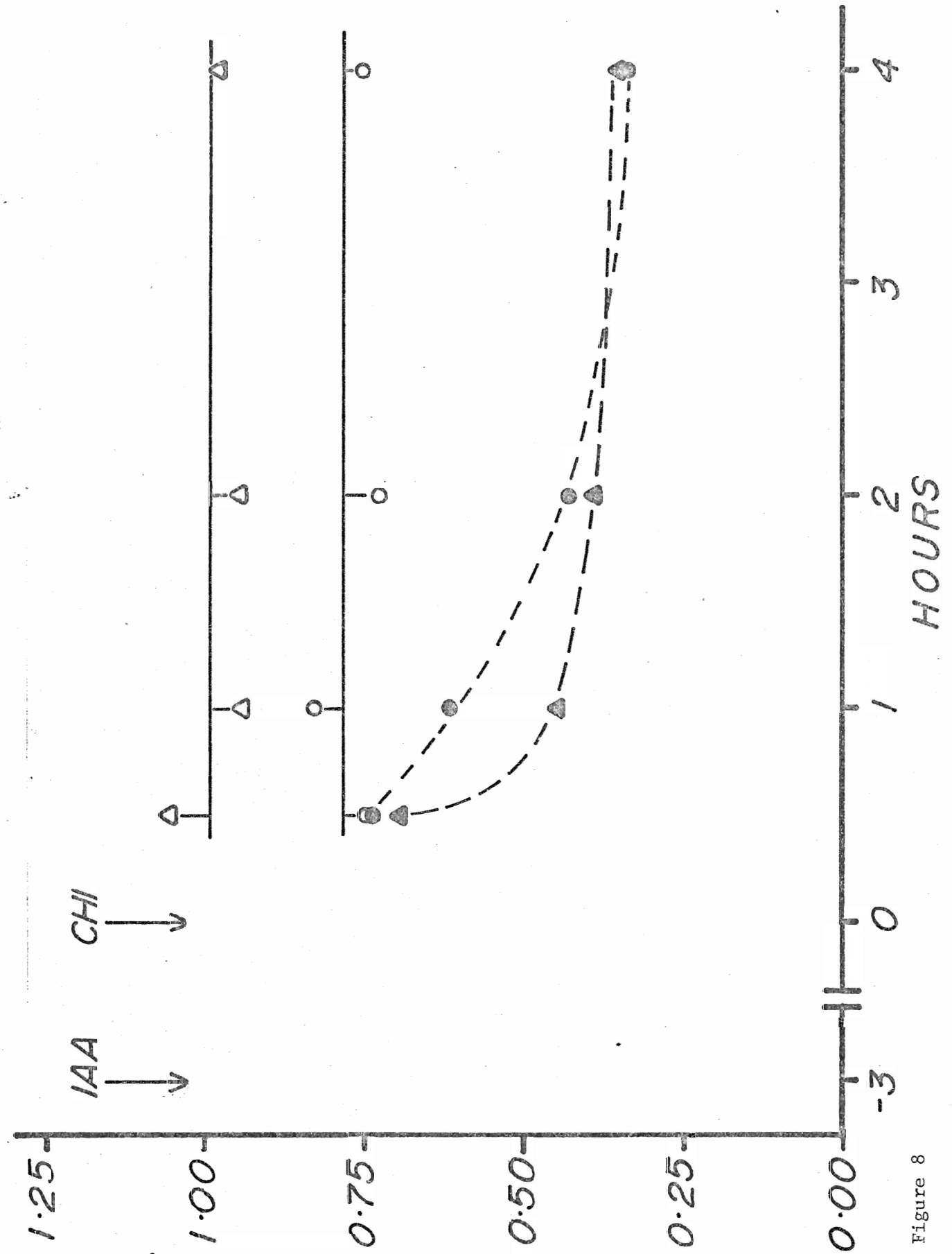

Dark $CO_2$ fixation rate
$C^{14} \times 10^5$ DPM / g fr wt / 10min
1·25
1·00
0·75
0·50
0·25
0·00
IAA
CHI
-3
0
1
2
3
4
HOURS

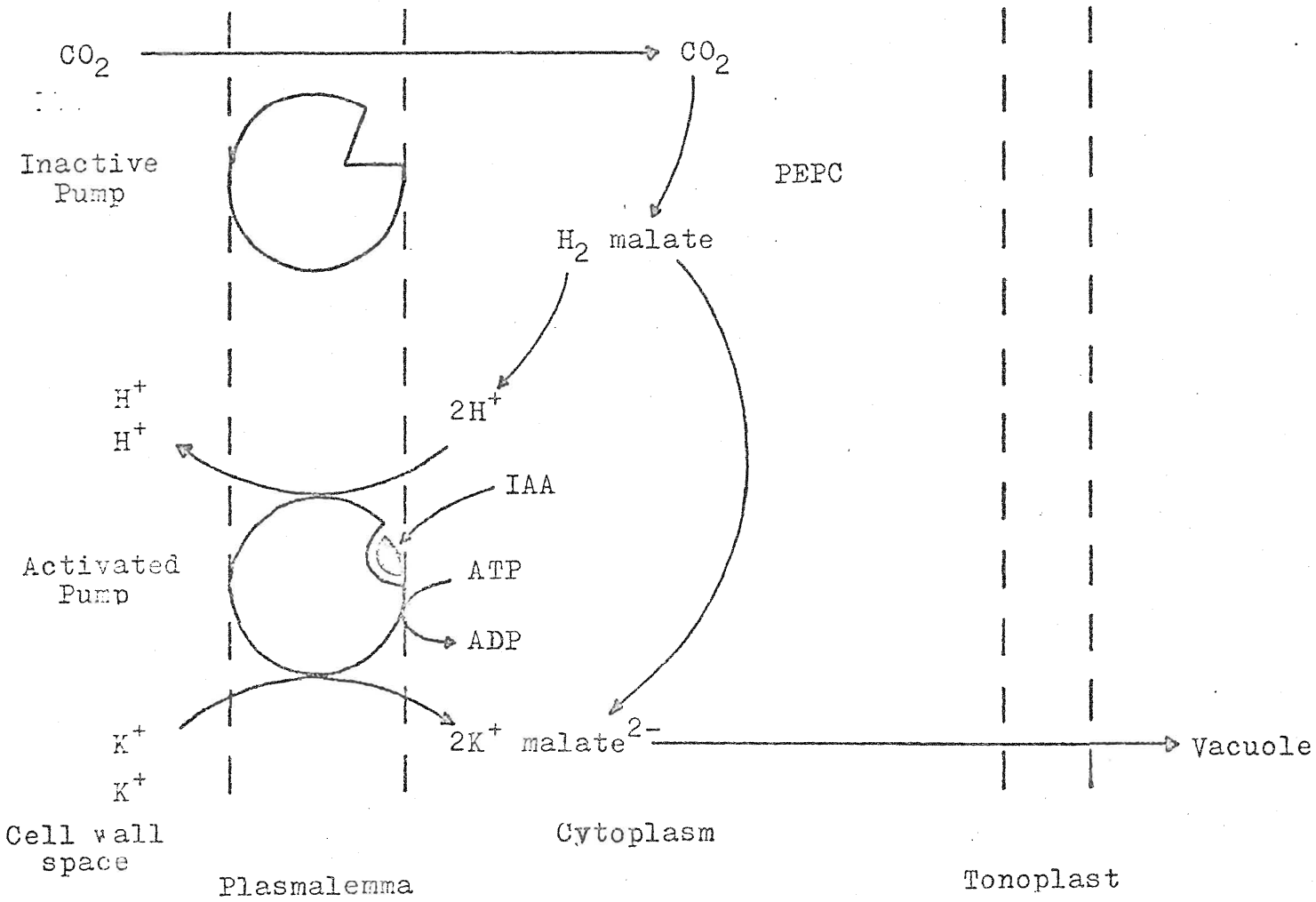
Figure 8

## Discussion

According to Haschke and Lüttge's model for IAA stimulated growth (Haschke and Lüttge, 1977b; Fig. 9), one may predict that the exclusion of $CO_2$ from the medium should decrease the IAA and FC stimulated growth substantially. However, contrary to the reports of Bown et al. (Bown et al., 1974, Dymock, Hill and Bown, 1977), no effect of 0.03% $CO_2$ on IAA and/or FC stimulated growth was observed in the present study (Table IIIa, Table IV and Fig. 5). Furthermore, the removal of $CO_2$ did not reduce coleoptile growth in the absence of auxin. Similarly, malate addition did not influence the growth rate of coleoptiles, irrespective of the presence or absence of IAA (Table V). In support of my results showing no $CO_2$ stimulation of coleoptile growth, the findings of the following authors may be cited:

Mer (1950, 1952, 1957) reported an inhibition of Avena coleoptile growth by 1%, 2.5%, 5% and 10% $CO_2$. Cockshull and Heath (1964) reported the inhibition of growth in wheat coleoptiles by various $CO_2$ concentrations up to 20% $CO_2$, both in the presence and absence of IAA. Harrison (1965a) in studying the effects of different $CO_2$ concentrations on growth of Avena coleoptiles, reported no detectable changes in growth between coleoptiles gassed with air and $CO_2$-free air. In $CO_2$ concentrations between 0.5% and 10%, the growth rate fluctuated during the entire period of experiments. The rates varied ±90% from the mean rate. Although it was concluded that 0.5%-10% $CO_2$ 'stimulated' growth, the large variations in his data do not permit such a conclusion. Leonard and Pinckard (1946) reported that a range

Fig. 9. A model for IAA-stimulated growth (Haschke and Lüttge, 1977b)



of $CO_2$ concentrations, between 0% and 15% had no appreciable influence on the elongation of cotton roots, and concluded that sufficient $CO_2$ could probably be produced within the cell from respiration for cellular growth. Nitsch and Nitsch (1956) presented evidence suggesting an enhancement of Avena coleoptile growth by $CO_2$ and that the optimum concentration of $CO_2$ was 5%. However, they also reported that their results with regards to $CO_2$ experiments were not always consistent. In short, the absence of influence by exogenous supply of $CO_2$ (0.03%) on growth in Avena coleoptile tissue is in good agreement with findings of the authors cited above.

An important point to be made about Haschke and Lüttge's model (Haschke and Lüttge, 1977b; Fig. 9), which emphasizes the importance of $CO_2$ fixation in IAA-stimulated growth, is the suggestion of an anaplerotic role of $CO_2$ fixation (Bown and Lampman, 1972; Bown and Aung, 1974; Splittstoesser, 1966). According to this suggestion, one might expect to observe a large stimulation of protein synthesis in FC-treated tissue, because of FC's ability to stimulate dark $CO_2$ fixation. However, in contrast to earlier reports (Bown and Aung, 1974a, Bown and Lampman, 1972), IAA, $CO_2$ and FC treatments failed to stimulate protein synthesis, as measured by precursor ($^3$H-leucine) incorporation into protein (Tables Ib, IIa, b and IIIb). Neither did I detect any substantial increase in the levels of protein due to these treatments. Moreover, employing the techniques and the experimental conditions as outlined in the Methods section, IAA and FC treatments had no detectable stimulation on the synthesis of RNA, at least during the first few hours (Tables VIb and c).

A lack of relationship between $CO_2$ presence and protein synthesis in the present system may be related to recent observations made in other laboratories (Haschke and Lüttge, 1977a, b; Stout, Johnson and Rayle, 1978). It was shown that the high level of malate, an early product of dark $CO_2$ fixation, remained unchanged for several hours, indicating that malate was not being used for biosynthesis of amino acid via the TCA cycle or other metabolic turnover processes (see Bown and Lampman, 1972). In addition, IAA had no influence on the incorporation of the label from $H^{14}CO_3$ into amino acids (Haschke and Lüttge, 1977a; cf. Bown and Lampman, 1971, 1972; Bown and Aung, 1974a). The authors (Haschke and Lüttge, 1977a, b; Stout, Johnson and Rayle, 1978) concluded that the accumulated malate could possibly be transported across the tonoplast into the vacuolar compartment along with $K^+$ or $Na^+$ ions as the divalent cation salt of malate where it would consequently maintain turgor pressure for the elongating cell. Therefore, the suggested anaplerotic role for dark $CO_2$ fixation in coleoptile growth may not be applicable in the present system.

The sensitivity of IAA-stimulated growth to actinomycin D and CHI treatments (Tables VIa; IIa) may simply indicate the absolute requirement of undisturbed transcription and translation processes for auxin enhanced growth (Nooden and Thimann, 1963), rather than auxin's promotion of these processes to induce elongation (Bown et al., 1974a, b). As proposed by Marré (1977a) and Lado et al. (1977), an already existing "short-lived protein" translated from an existing short-lived mRNA may play an important role in the chain of events mediating the effects of IAA. Ray (1977a, b) has also suggested the possibility for the involvement of secretory proteins (at the ER) in IAA-induced $H^+$ ion secretion and growth. Marré

(1977a) proposed that the synthesis of that particular "short-lived protein" would be independent of the presence of IAA. Alternatively, IAA may specifically induce the syntheses of the "short-lived" RNA(s) and protein(s) "essential" for auxin-stimulated growth (Key and Ingle, 1964; Bown and Aung, 1974a; Marré, 1977a). Even if auxin promotes the synthesis or turnover of RNA and protein(s), they may comprise only a small portion of the total RNA or protein content of the cell and, hence, may be difficult to measure (Nooden and Thimann, 1963; Venis, 1964).

A 3 h pre-treatment with actinomycin D did not substantially influence growth of control tissue in the following 6 h, whereas this pretreatment did reduce the IAA-stimulated growth to nearly the growth rate of non-IAA treated tissue (Table VIa). On the other hand, both the control and IAA treated tissue ceased to grow during a 4 h exposure to CHI (Table Ia, IIa). A much more pronounced effect of CHI on the growth process may be related to its rapid inhibitory action on several metabolic processes which accompany the inhibition of protein synthesis (see Table VII and references cited therein; also, Cocucci and Marré, 1973; Lado et al., 1977 and Van Steveninck, 1976). The consistent observation of only partial inhibition of FC-stimulated growth by the inhibitors of RNA and protein synthesis (Act D and CHI, respectively) is in good agreement with reports for pea internode segments (Lado et al., 1973; 1977; Marré, 1977a, b). This is probably due to the different action of FC, involving the plasmalemma (Dohrmann and Hertel et al., 1977; Beffagna et al., 1977; Marré, 1978), as opposed to the varied and complex control points (e.g., endoplasmic reticulum, plasmalemma, Golgi apparatus) suggested for auxin (Ray, 1977a, b; Morre and Mollenhauer, 1976; Robinson, 1977; Chrispeels, 1976; Marré,

Table VII The effects of cycloheximide on some important processes stimulated by IAA and FC

| | Degree of inhibition by CHI (high or low) | | | References |
|---|---|---|---|---|
| | Control | IAA | FC | |
| $H^+$ ion secretion | high | high | low | Cleland, 1976a<br>Rayle, 1973 |
| Hyperpolarization of the transmembrane electric potential | high | high | low | Depolarization<br>Marré *et al.* 1974b, c. |
| Accumulation of malate | high | high | low | n. mol. malate<br>Stout *et al.* 1978 |
| Growth | high | high | low | Marré 1977b<br>also, see Table IIa |

1977a; Darvill et al., 1977). Therefore, it may be concluded that FC-stimulated growth is less dependent on the continual synthesis of RNA and proteins, in contrast to auxin-stimulated growth.

In the present investigation, the radioactivity in proteins and in the ethanol soluble fraction were consistently higher in FC-treated tissue when CHI was absent (Tables Ib and IIb). The addition of CHI inhibited the incorporation of radioactivity from $^{3}$H-leucine into proteins by approximately 60-70% irrespective of the presence of IAA and/or FC, although it had little influence on the uptake of leucine as measured by the total uptake of radioactivity (Tables Ib and IIb). Consequently, the specific radioactivity of protein in FC-treated tissue was high in the absence of CHI and the addition of CHI caused the accumulation of radioactivity in the tissue. On the contrary, IAA had an inconsistent influence on the incorporation of radioactivity from $^{3}$H-leucine into protein, in the absence of CHI. However, one might argue that, in some cases, the radioactivity in protein was slightly higher in IAA or $CO_2$-treated tissue than in the control tissue (Tables IIIa, b), but these occurred with marginal increases in the ethanol soluble radioactivity. Consequently differences may be due to variable responses (or fluctuations) among different batches of tissue or to an influence of IAA or $CO_2$ on protein synthesis. Therefore, it may be concluded that the incorporation of $^{3}$H-leucine into protein and into tissue, in response to IAA or $CO_2$, was not consistent (Tables IIb, IIIb). Similar insignificance was also reported by Nooden and Thimann (1963) and Bates and Cleland (1976*) for Avena coleoptile tissue treated with IAA. On the other hand, Splittstoesser (1966) reported a stimulation of protein synthesis by atmospheric $CO_2$ in non-photosynthetic tissue, tomato roots.

Lado et al. (1977) reported that in pea stem sections, a 30 min incubation with $^{14}$C-leucine resulted in a 13% or 52% increase in the TCA

*Bates, G. and R. Cleland. 1976. Protein synthesis during auxin-induced cell elongation. Plant Physiol. 57: S87.

(trichloroacetic acid) soluble radioactivity in response to 20 μM IAA and 20 μM FC, respectively; at the same time, the incorporation into the TCA insoluble fraction was increased by 6% and 18% for IAA and FC treated tissue (Lado et al., 1977). The interpretation of the data by the authors was that both IAA and FC stimulated the uptake of leucine into tissue but that they (IAA and FC) had little influence on the rate of leucine incorporation into proteins. On the other hand, some increase of radioactivity, due to IAA, in both the ethanol soluble fraction and proteins (56% and 59%, respectively, measured over a 5 h period) was reported for the same tissue (i.e., pea stems) (Nooden and Thimann, 1963). Since IAA did not change the protein content considerably (up to 29 h), the authors (Nooden and Thimann, 1963) concluded that the observed higher radioactivity in proteins was due to the stimulated uptake of amino acid.

A disagreement exists between this study and the work of Lado et al. (1977). In their system (pea stem sections), cycloheximide at a concentration of 5-100 μg/ml not only inhibited the incorporation of $^{14}$C-leucine into protein by over 90%, but also inhibited the uptake of leucine into tissue by about 30%, regardless of the presence of IAA and FC. In our system (Avena coleoptiles), although the 10 μg/ml cycloheximide caused a general decrease of the overall radioactivity in the tissue, i.e., a total amount of radioactivity in protein and ethanol soluble fraction decreased, it did not change, significantly, the amount of ethanol soluble radioactivity as clearly observed in the control and IAA-treated tissue (Tables Ib and IIb). The ethanol soluble radioactivity in FC-treated tissue was considerably higher in the presence of CHI, as mentioned earlier, probably because of FC's ability to enhance the uptake of the amino acid (Tables Ib and IIb). Therefore, according to Lado et al. (1977), CHI caused the

inhibition of leucine uptake into tissue, whereas in the present study, no such effect by CHI was observed. The observations made by Lüttge et al. (1974) appeared to support the data from the present investigation. These investigators reported that CHI had no influence on the membrane transport process controlled by plasmalemma or tonoplast; however, protein synthesis is a basic requirement for the symplasmic transport from cell to cell via the plasmodesmata (Lüttge et al., 1974).

It is interesting to compare the effects of CHI on some of the processes stimulated by IAA and FC, namely, $H^+$ ion secretion, hyperpolarization of the transmembrane electric potential, synthesis and accumulation of malate and growth. As may be seen in Table VII, cycloheximide exhibited a differential inhibition on these processes which are stimulated by IAA or FC.

In the absence of CHI, both IAA and FC stimulated the appearance of label in Avena coleoptile tissue supplied with $^{14}C$-bicarbonate (Johnson and Rayle, 1976). The majority of the label fixed was in the form of malate (Johnson and Rayle, 1976; Haschke and Lüttge, 1975, 1977). Such an accumulation of malate in IAA and FC treated tissues may result from different mechanisms. First, the enhanced extrusion of hydrogen ions (by IAA and FC) may result in the rise of cytoplasmic pH, which would stimulate the activity of PEP carboxylase, as explained by the pH-stat mechanism (Davies, 1973; Raven and Smith, 1974; 1976); consequently, the rate of malate synthesis would be increased. Secondly, the accumulation of malate may also result from an increase in the level of cytoplasmic bicarbonate through increased respiration and/or the enhanced uptake of $CO_2$ (Johnson and Rayle, 1976; Marré, 1977b). In Figure 8, it is observed that, in the

absence of CHI, IAA stimulated the rate of bicarbonate fixation by approximately 25% that of the control rate. CHI, which inhibited the IAA-stimulated $H^+$ ion secretion within 10 minutes (Rayle, 1973), also inhibited the IAA-enhanced rate of bicarbonate fixation within 30 min. By 2 h, $CO_2$ fixation rate in the tissue treated with CHI was approximately 50% that of the control rate. Stout, Johnson and Rayle (1978) also reported the inhibition of IAA-enhanced malate accumulation by CHI, over a 2 h period. The simplest explanation one may provide would be that the inhibition of $H^+$ ion extrusion by CHI may result in a decrease in the cyotplasmic pH, which would consequently lower the activity of PEP carboxylase and thus "turn off" the synthesis of malate. However, the specificity and the rapid action of the inhibitor (CHI) on protein synthesis and the subsequent complications, such as impaired respiration (Cocucci and Marré, 1973) may also be noteworthy.

The results outlined in this thesis are consistent with recent reports concerning IAA and FC stimulated growth. The stimulation of leucine uptake by FC reflects similar reports showing that in pea stem and maize root segments, FC stimulates the energy dependent uptake of cations, anions, amino acids and sugars (Marré, 1977b). Marré (1977b) suggests that transmembranous hyperpolarization may serve as the driving force for uptake of these substances. The data in Figure 8 indicate that IAA stimulates $CO_2$ fixation and that fixation is inhibited within 30 min by cycloheximide. This result extends the findings of Stout, Johnson and Rayle (1978) that IAA stimulated malate accumulation is inhibited by cycloheximide within 2 h. In addition, work with pea internode segments demonstrated that, as in this study, IAA stimulated

growth was much more sensitive to CHI than FC stimulated growth (Lado et al., 1977). The different sensitivities of FC and IAA stimulated growth to CHI and actinomycin D, and the inability of IAA to mimic FC in stimulating leucine uptake suggest fundamental differences in the mechanisms of growth promotion by these two agents. In this respect, it is interesting to note that substantial evidence suggests that FC acts directly on the plasmalemma (Dohrmann and Hertel et al., 1977; Beffagna et al., 1977; Marré, 1978), whereas the ER membrane has been suggested as the site of IAA action (Ray, 1977a, b). Ray (1977a) suggests that "Combination of auxin with its receptor sites of the ER could induce $H^+$ transport from the cytoplasm into the ER cisternal space. The acid contained therein would be transported along with secretory proteins contained in the ER space, to the cell exterior (cell wall space), probably via the Golgi system". If protein secretion and $H^+$ secretion are coupled processes, cycloheximide would inhibit not only protein synthesis but $H^+$ secretion and growth.

The results in this thesis support proposals that fusicoccin acts by combining with a plasmalemma binding site to stimulate $H^+$ secretion in a process which is independent of protein and RNA synthesis (Marré 1977a, b). The ability of fusicoccin to stimulate leucine uptake indicates a change in the function of the plasmalemma if it is assumed that leucine accumulates in the cytosol. On the other hand, the sensitivity of IAA stimulated growth to inhibitors of RNA and protein synthesis, and the inability of IAA to stimulate leucine uptake are consistent with Ray's proposal (Ray, 1977a) that the binding site is on the ER and that protein secretion and $H^+$ secretion are coupled processes.

# CONCLUSION

20 μM IAA and/or 1 or 10 μM FC stimulated growth of Avena coleoptile tissue (Figs. 3, 5, Tables Ia, IIa and IIIa). However, FC and IAA, as indicated by experiments involving the incorporation of labelled precursors, do not stimulate the synthesis of RNA or protein during the initial hours of growth promotion (Tables IIb, IIIb and VIb and c). Actinomycin D or cycloheximide which inhibited RNA and protein synthesis respectively, inhibited IAA stimulated growth completely, but only partially inhibited FC stimulated growth (Tables Ia, IIa and VIa). Therefore, in contrast to IAA stimulated growth, FC stimulated growth appears less dependent on the continual synthesis of RNA and protein (Tables Ia, IIa and VIa). The data also strongly suggest that FC, unlike IAA, stimulates the uptake of $^{3}$H-leucine into coleoptile tissue. These data are consistent with results suggesting that the site of action for growth stimulation by FC is the plasma membrane (Marré, 1978). The hyperpolarization of the membrane potential in response to FC stimulated $H^{+}$ excretion may serve as the driving force for FC stimulated leucine transport (Marré, 1977a).

These differences between FC and IAA stimulated coleoptile growth support suggestions that FC and IAA stimulate growth through different mechanisms (Marré, 1977b). It has been suggested that the site of action of FC is the plasma membrane (Beffagna et al., 1977; Dohrmann et al., 1977; Marré, 1977a) and the site of action for IAA is the ER (Ray, 1977a; Marré, 1977b).

IAA stimulated the fixation of labelled $^{14}C$-bicarbonate by coleoptile tissue by approximately 25% (Fig. 8). The addition of cycloheximide, that was reported to inhibit $H^+$ secretion (Rayle, 1973), inhibited the fixation of labelled bicarbonate by coleoptile tissue within 30 min. Therefore, although dark $CO_2$ fixation appears to be involved in IAA stimulated growth, $CO_2$-free air did not decrease IAA or FC stimulated growth (Tables IIIa and IV, Fig. 5). In addition, 1 mM malate, pH 6.0 exhibited a similar absence of influence on growth, in the presence or absence of IAA (Table V). Therefore, these data contradict the earlier reports concerning the influence of $CO_2$ or malate on growth. The dark fixation of $CO_2$ may serve to generate $H^+$ ions during growth process (Haschke and Lüttge, 1977b; Hill and Bown, 1978, Marré, 1977a). The accumulated potassium salt of malate may provide turgor pressure and may also maintain the electrochemical balance of the cell (Haschke and Lüttge, 1975, 1977b; Stout, Johnson and Rayle, 1978).

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